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PROCEEDINGS OF THE EUTROPHICATION-BIOSTIMULATION ASSESSMENT WORKSHOP HELD AT HOTEL CLAREMONT, BERKELEY, CALIFORNIA ON JUNE 19-21, 1969

E. J. Middlebrooks, et al

Federal Water Pollution Administration
Corvallis, Oregon

1969

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Held at
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PROCEEDINGS

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ASSAY PROCEDURES SESSION

Moderator: Warren J. Kaufman
Professor of Sanitary Engineering
and Associate Director,
Sanitary Engineering Research Laboratory
University of California, Berkeley

V

WHY WE ARE HERE

A. F. BARTSCH

Director,
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This meeting, the Eutrophication-Biostimulation Assessment Workshop, is the first of four workshops to be held at strategic places in the United States. Frankly, the format and mechanics of this workshop are an experiment, and from this trial experience the nature of future workshops will be determined.

It is my task at the moment to tell you why we are here. There are several reasons, and I wish to mention each of them briefly.

Across the Nation wherever there are surface waters there are increasing public demands for preventive and remedial actions to curb the velocity of eutrophication processes. It is not surprising, therefore, that recent sessions of the Congress have resulted in specific bills to support research and demonstrations in preserving lakes. There is great public awareness of the existence of eutrophication as a problem and the part that people play in accelerating it. There is reason to believe that eutrophication is the most serious and pressing water quality problem we face in the future. This is one reason why we are here.

Impressive scientific resources of the Nation already are directed to the eutrophication problem. Represented are scientists on government rolls as well as in research laboratories of universities, foundations, and profit organizations. The principal areas under scientific scrutiny are ones that relate to: 1) understanding the problem, 2) ecological control, 3) mechanical control, 4) chemical control, and 5) biological control. To the question: How many people are now participating in this effort? — we must respond by saying we do not exactly know. Similarly, there is no precise knowledge on the total number of dollars expended in this effort. But I can point out that the Federal Water Pollution Control Administration is spending about $2 million during the present fiscal year for in-house and grant- and contract-supported research projects.

To reach this point in program delineation, the obvious scientific ideas have already been put into motion. There is need to go beyond this. Pressing at the moment is the need for new scrutiny, new thought, new ideas on where we go scientifically from here. That, also, is why we are here.

In spite of the pleasant surroundings here in the Bay Area, you are not here to be entertained. You are knowledgeable in the subjects under consideration. Certainly you have new ideas and we as a group here collectively need them. This is not to be a symposium or seminar but a workshop in which everybody works. While there are presentations on the agenda, their purpose is to serve as a stimulus for discussion to coax out ideas. Toward this end, your participation is needed. That, then, really is why we are here.

NEED FOR ASSAYS

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During the past decade we have witnessed unprecedented activity directed toward an understanding of the enrichment process which occurs in lakes, streams, and estuaries. Despite this activity, the interrelationships of climatic, physical, chemical, and biological factors influencing biological productivity in the aquatic environment are not understood clearly nor has there been developed a "standardized" bioassay for determining the enrichment level or fertilizing potential of various waters. This is obviously not surprising in view of the complexity of the interrelationships and the wide variations in environmental conditions, not only geographically, but even within a single aquatic system. Nonetheless, in view of the current awareness, concern, and desire for improvement of the quality of our water resources, it is imperative that scientists and engineers in the field of water quality management not only refine present analytical methods to quantify water quality, but also develop new "tools" to evaluate existing quality and the effectiveness of corrective measures.

The use of bioassays is not novel in this field and although a major effort has been directed toward bioassays to test for the influence of toxic agents and the effects of pollution on biological activity, substantial work has been done to evaluate the potential fertility of water.

Of particular value with regard to biological assays are the transactions of the seminars on biological problems in water pollution held at the R. A. Taft Sanitary Engineering Center in recent years (Tarzwell 1957, 1960, 1965). Perhaps more directly related to the subject to be discussed at this meeting are the reports by Skulberg (1964, 1967) of his work in Norway. The history of the use of laboratory cultures of algae to assess fertility has been received by Skulberg (1964) and his development and practice of a bioassay procedure for such use is no doubt familiar to this group. Despite the progress that has been made, however, there remains the need for standardization of algal growth tests that will hopefully become acceptable to the increasing number of research and operating personnel concerned with this problem.

As pointed out in the Provisional Algal Assay Procedure proposed under the sponsorship of the Joint Industry/Government Task Force on Eutrophication, a "standard method" is needed for use:

"1. as a bioassay for available algal nutrients in the absence of toxicants;

2. to estimate the effect on algal growth of adding extraneous substances to water;

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3. to determine the levels to which nutrients in a given body of water must be reduced to limit algal growth to acceptable levels."

Skulberg (1964) notes that the bioassay method may be of additional help concerning the assessment of the effects of pollution on eutrophication and may result in a useful parameter for comparative limnology, for defining population equivalents, and for testing the effect of sewage-treatment plants in reducing the component of mineral nutrients in the effluents. Certainly, as pointed out by Skulberg, bioassay procedures are needed as a supplement to chemical and physical analyses for the proper evaluation of eutrophication problems and corrective measures.

The recognition of eutrophication as a pressing environmental problem makes it incumbent upon scientists and engineers in the water resources field to work toward standardization of procedures that can be used to obtain reproducible and comparable results.

In the development of standards for water quality, a major step is in the identification and quantification of quality. (See Figure 1, based on the report of the Committee on Pollution — National Academy of Science — National Research Council, 1966.) Without dependable and acceptable methods, standards can neither be intelligently formulated nor successfully enforced.

During the next few years, millions of dollars in public funds will be expended as programs dealing with nutrient control to receiving bodies of water are implemented. If these funds are to be spent wisely, priorities must be established with regard to kinds and amounts of nutrients that must be controlled if maximum effectiveness is to result. To do this, it will be necessary to develop both laboratory and field bioassay procedures that are reliable and reproducible, and hopefully relatively simple. It is obvious to those of you engaged in this seminar that this will not be an easy task, but the discussions that follow not only will point out the difficulties but will also indicate the progress that has been made.

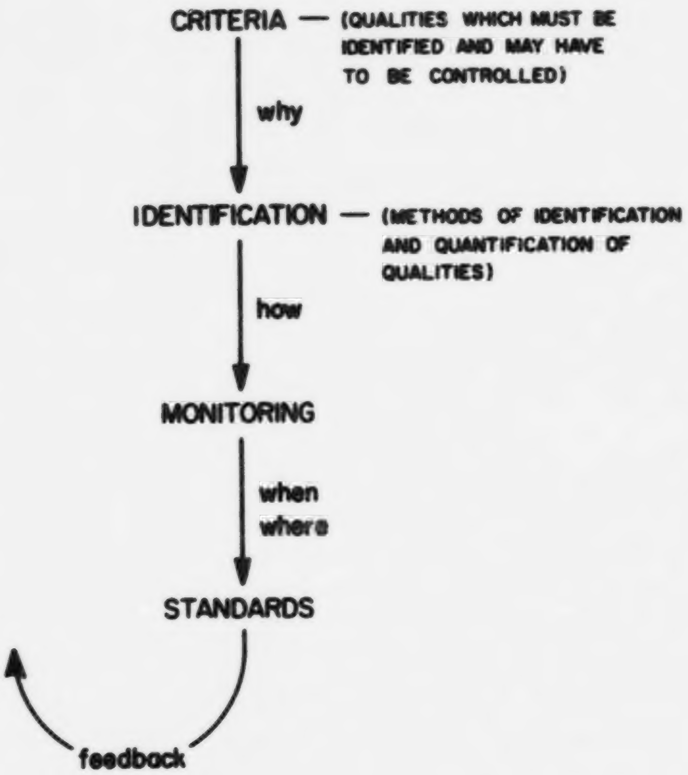
CRITERIA — (QUALITIES WHICH MUST BE IDENTIFIED AND MAY HAVE TO BE CONTROLLED)
why
IDENTIFICATION — (METHODS OF IDENTIFICATION AND QUANTIFICATION OF QUALITIES)
how
MONITORING
when
where
STANDARDS
feedback

FIGURE I. MAJOR STEPS IN IDENTIFICATION AND QUANTIFICATION OF WATER QUALITY

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CONTINUOUS-FLOW (CHEMOSTAT) ASSAYS

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INTRODUCTION

Although the use of continuous-flow cultures of organisms date from earlier in this century (Malek and Fencl 1966), their full development for the study of microorganisms awaited the mathematical framework provided by Monod (1950) and Novick and Szilard (1950). Since then, Herbert et al. (1956) have furnished a very clearly written and well-documented paper from which an intuitive understanding of the process is easy to obtain, and Malek and Fencl (1966) have presented the most complete, recent publication on continuous-flow cultures. The adaptation of these and other theoretical developments to natural ecosystems has been suggested by Dugdale (1967) and applied to batch and continuous-flow assays of biostimulatory response by Pearson et al. (1969).

Previously, basic research on continuous-flow systems has been used to analyze some aspects of algal growth. For example, in regard to light spectral qualities (Oswald 1957, Shelef et al. 1968) and light intensity (Fujimoto et al. 1956, Phillips and Myers 1954) and the effects of self-shading (Pipes and Koutsoyannis 1962) considerable information has been obtained. Temperature effects and their interactions with light and nutrient concentrations and resultant effect on growth have been studied by Maddux and Jones (1964). In bacterial cultures, magnesium (Tempest et al. 1965, 1967) and phosphorus (Slezák and Sikyta 1967) have been studied in regard to their effect as limiting nutrients in population growth. Nitrate limitation in Chlorella and the effect on growth (Shelef et al. 1968) have been studied in relation to light. Droop (1966) has determined Vitamin B_{12} limitation of the growth of the chrysomonad, Monochrysis lutheri. The effect of added CO_2 on growth of Chlorella at various residence times has been observed to follow the expected utilization of limiting concentrations of inorganic carbon (Pipes 1962). Jannasch (e.g., Vaccaro and Jannasch 1966), studying bacteria, has measured heterotrophic growth on organic substrates in sea water using continuous flow systems. These results indicate the wide range of data obtained from continuous flow systems which is available in the literature and which is directly concerned with analysis of limiting factors on the growth of microorganisms.

Features of Continuous-Flow Systems

Several basic requirements necessary to obtain steady state for single-reactor, continuous flow systems are listed as follows: 1) inflow equal to outflow; 2) constant flow rate and volume; 3) complete mixing and uniform dispersion of the microorganisms; and 4) maintenance of a uniform environment. Two common systems utilized in studies of growth of microorganisms are the chemostat and the turbidostat. The chemostat maintains constant flow rate and operates best at flow rates considerably less than the washout rate, i.e., where the population of cells will not wash out as a result of small variations in flow rate. The turbidostat maintains constant turbidity by adjustment of flow rate and operates best at high growth rates where for small changes in flow rate measurable differences in cell density occur. Chemostats are more convenient and economical and hence discussion will be limited to consideration of chemostats, although equivalent results are theoretically obtained from each (Herbert 1958).

In the chemostat the volume is fixed and the flow rate adjusted to a constant rate so that a desired residence time in the reactor (culture flask) is maintained. The initial concentrations of nutrient in the reactor are the same as the influent concentrations and the starting population of microorganisms, the algal (in our case) seed is less than the final steady state concentration. The seeded algal population increases and after some time attains a steady state concentration where the following conditions hold: 1) the concentration of cell mass in the reactor and in the effluent remain constant, 2) the average specific growth rate is constant, 3) nutrient concentrations are constant, and 4) cell and nutrient concentrations are in constant proportion to the external environment. Hence, a chemostat provides a culture of microorganisms at steady state which are theoretically maintained under a constant physical environment and is nonvariant chemically and biologically. The cells in a completely mixed system will be diluted or washed out at a fixed rate dependent on the flow rate and the volume of the reactor. For a steady state to exist the cell population must grow at a rate adequate to replace those cells which are washed out of the system. Growth can take place only by utilization of energy and this growth can be controlled by some nonenergy limiting factor. Hence, growth rate will be some function of the concentration of the limiting factor.

A certain portion of the energy of a cell is utilized for maintenance reactions; hence, in compensating for maintenance this energy expended must be considered in measuring the growth rate. Substrates are defined as those limiting substances which are a source of energy (organic material, light) and nutrients as those limiting substances which are not a source of energy (CO_2, N, P, trace materials) and which may in fact require energy in order to be utilized. Hence the maintenance requirement may represent a significant portion of an energy balance when substrates limit growth but not significantly affect the biomass level when the energy source is in excess and nutrients limit growth.

As an example of a chemostat experiment some idealized curves showing the relative change in algal cell material and limiting nutrient (phosphorus) concentration for a chemostat are shown in Figure 1. As can be seen, the algal concentration increases to a maximum steady state level which can theoretically be maintained indefinitely. The phosphorus concentration decreases rapidly to a low concentration and this also can be maintained at that level indefinitely.

The phosphorus concentration in the reactor would be equivalent to the influent concentration except that the cells utilize phosphorus and hence remove it from solution. For comparison the calculated phosphorus concentration, based on a constant yield of cells per unit phosphorus removed from solution, is shown. The actual concentration decreases at a rate faster than calculated, indicating that the yield or, inversely, the cell concentration of phosphorus is not constant throughout the transient stages of the experiment. At steady state and phosphorus limiting growth, a constant yield would be expected. As a result the phosphorus concentration would be at a low level. If the steady state nutrient concentration in the reactor is not at a low level and the culture is not operated at residence times near

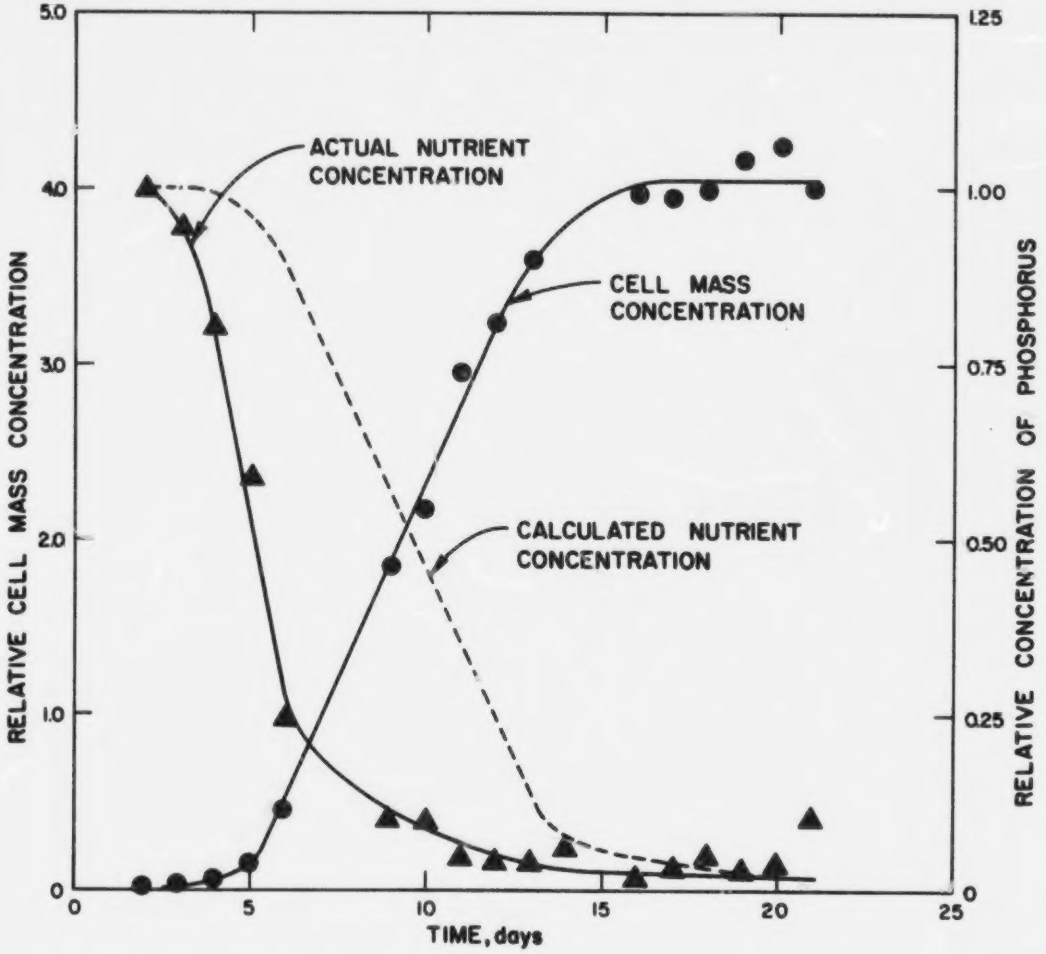

FIGURE I. RELATIVE CHANGES IN ALGAL BIOMASS AND PHOSPHORUS CONCENTRATIONS IN A CHEMOSTAT AS STEADY STATE IS APPROACHED

washout, then the nutrient of interest is probably not limiting. However, as shown in Figure 1, the converse is not true. Accumulation in excess of what is needed apparently occurs and hence low concentrations are not evidence that the specific nutrient controls growth.

Essentially the nutrient is completely utilized. This steady state pattern, i. e., nearly complete utilization of nutrient, is similar for any residence time up to the minimum residence time prior to "washout" of cells and for any influent concentration until a concentration is reached at which that nutrient is no longer limiting (Herbert 1956).

If it is true that the only limiting factor is the nutrient of interest, then it is theoretically possible to utilize any reactor shape, influent and effluent design, initial nutrient concentration, and physiological state of the seed population, and the final steady state conditions will be unaffected. That is, the level of biomass supported is a function of the nutrient concentration and specific growth rate; in turn both of these variables are fixed by the hydraulic residence time and the influent nutrient concentration. Hence, reactor design has largely been a matter of convenience and dependent on the specific use of the continuous culture. As a result, many different designs have been suggested in the literature (e. g., see Myers and Clark 1944; Malek and Fencl 1966; Shelef et al. 1968). However, it is logical that the design be as simple as possible yet consistent with the concept that other factors not limit growth (e. g., light penetration) or interfere with operation of the chemostat (clogged orifices, unmixed areas in the reactor, wall growth). A very simple design has been suggested for the growing of algae in the development of algal assay procedures which attempts to satisfy these requirements (Provisional Algal Assay Procedures 1969).

The Chemostat as a Technique for Analyzing Samples of Natural Aquatic Ecosystems

Samples for assay are collected and brought into the laboratory where the appropriate analyses and preparations for introducing the sample to the chemostat are made. However, for any type of bioassay several natural factors must be considered when one collects a sample from a lake or stream and brings it into the laboratory for bioassay. In the laboratory the sample is treated (filtered) to remove interfering organisms and the assay begun using a suitable test organism. Then, under continuous light at a suitable intensity and at a constant temperature, the algal growth is determined for the water sample. Hence, microorganisms are removed from the sample as well as some nutrients which are filterable; day-night cycling and variation in light intensity and temperature are not maintained; and the sample is assayed in an unnatural environment. The factors impinging on the sample can be divided into two groups as shown in Table I.

To determine the response of algal organisms to what is actually in the water, it is necessary to eliminate the effect of extrinsic factors and insure that only those factors in the sample are assayed. Hence, light and temperature are optimized, mixing is maintained, and CO_2 (in air) is added to the sample during the assay. Despite the fact that the extrinsic factors may control productivity in nature, the assay can be related only to those factors which limit growth in the sample. Although some continuous-flow work has been done on mixed cultures of organisms (Tempest et al. 1967), it is still preferable at this time to make bioassays with unialgal cultures to maintain comparability and, hence, sacrifice a relationship with the environment in determining the algal response to nutrient and toxicant concentrations and their chemical milieu. The results gained from such an assay then apply only to those intrinsic factors and may not relate to what actually limits the growth rate and standing crop of algae in nature.

TABLE I

FACTORS WHICH AFFECT THE GROWTH
OF ALGAE IN A SAMPLE OF WATER

Properties of the Environment (extrinsic factors)

- Light
- Temperature
- Hydraulics of the aquatic system
- CO_2
- Presence of other organisms such as decomposers and predators

Properties of the Sample (intrinsic factors)

- Chemistry of sample (e.g., organic material, salts, alkalinity, pH)
- Concentration of nutrients
- Concentration of toxicants
- Concentrations and types of organisms present

It is not recommended at this stage of development that one should model natural aquatic ecosystems by direct application of the kinetic theory as used in chemostat work. However, combinations of various continuous-flow and transient models can be applied to natural systems to help determine the appropriate factors which control productivity in nature. The techniques used for deriving the theory of continuous-flow cultures, i.e., mass balances and kinetics of algal growth, are important tools of research which should be applied to natural ecosystems in order to gain useful information on productivity, nutrient cycling, and successional relationships. This does not preclude use of the chemostat in bioassays. The disadvantages of using a complex and time-consuming technique such as a chemostat are offset by the increased information and the advantages of measuring algal response parameters in a dynamic, open system at steady state.

USE OF CHEMOSTATS FOR ASSAYING BIOSTIMULATORY RESPONSE

Three aspects of the problem of assaying biostimulatory response in relation to the natural environment are of considerable importance. With respect to the use of chemostats, some results are discussed below with hopes of identifying some of the factors involved with the following: 1) assay of a sample containing several nutrients which are approximately equally limiting to growth and the resultant problems of identifying the limiting nutrient, 2) use of natural algal populations as opposed to seeded species, and 3) the problem of washout of the algal population at low nutrient concentrations.

Multiple Limiting Nutrients*

Several nutrients will limit growth at the same time in most water samples because the concentrations of nutrients will be at levels that are equivalently related to growth, e.g., iron and phosphorus have both been shown to stimulate growth of a test alga in water samples of a marl lake (Eyster 1958). There would be considerable difficulty in interpreting algal growth in samples of lakes such as these. An example of how algal growth is affected by two different factors is found in a chemostat analysis of how limiting orthophosphate concentrations control the growth of the green alga, Selenastrum capricornutum, Printz. The four reactors were pyrex flasks each containing one liter of culture medium and each with a different phosphorus concentration. The medium was a modification of Skulberg's Z-8 (1966) and was composed so that the highest concentration of the four phosphorus concentrations was only one-fifth of the normal concentration (in ratio to the other constituents). The inflow and outflow of fluid was controlled by continuous operation of peristaltic pumps and mixing was accomplished by shaking vigorously on a large shaking table. Incident light was approximately 8000 lux (860 ft-candles) at the surface and the temperature of the cultures was approximately 21-22^{o}C. During Experiment 1 some air (hence CO_2) diffused into the culture flask but this was only at a slow rate. Hence, for at least the higher phosphorus concentrations, population growth was controlled by CO_2 concentrations. Afterwards, in Experiment 2, a mixture of CO_2 and air was blown over the surface of the culture to add CO_2. Because the pH of the culture was controlled by free CO_2 in solution, pH was used as an index for determining adequate CO_2 concentrations in the culture. The flow of CO_2 was increased when the pH increased to 8.5 or higher and decreased when the pH fell below 7.

Some results of these experiments are shown in Figure 2 where cell concentration is plotted against time for the chemostats receiving the highest nutrient concentrations. As can be seen, relatively stable, steady states were obtained for both cultures but the level of cells supported was considerably less (about 20%) in the culture from Experiment 1. Also the comparison between the two levels of pH indicates that it is CO_2 that limits growth.

Results obtained for the four different phosphorus concentrations are shown in Figure 3. The phosphorus utilized is equal to the influent concentration minus the reactor concentration. Cell mass was estimated by COD determinations. For the low concentrations of phosphorus very scattered results were obtained and it was difficult to maintain steady state. However, for the two higher concentrations reasonable steady states were obtained and the values plotted with an expected intercept of zero, i.e., no growth would be expected for zero utilization of phosphorus. In Experiment 1 the results were difficult to interpret. Although in one case the cell concentration was quite a bit higher than its counterpart in Experiment 2, the COD was considerably lower. At the highest concentration the COD and cell count were both considerably lower for Experiment 1 than for Experiment 2. In general, the cells were considerably smaller in Experiment 1. Also, as can be seen in comparing the two experiments, not all the phosphorus was utilized at the highest concentrations of phosphorus for Experiment 1, further indicating that CO_2 was controlling the growth. The results indicate that with two-factor limitation

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*This work was accomplished at the Norwegian Institute for Water Research, Oslo, with the considerable advice and help of Olav Skulberg and the technical help of Josef Kotai, and Mrs. Unni Ephraimson, and with the financial aid of a Fulbright Graduate Fellowship.

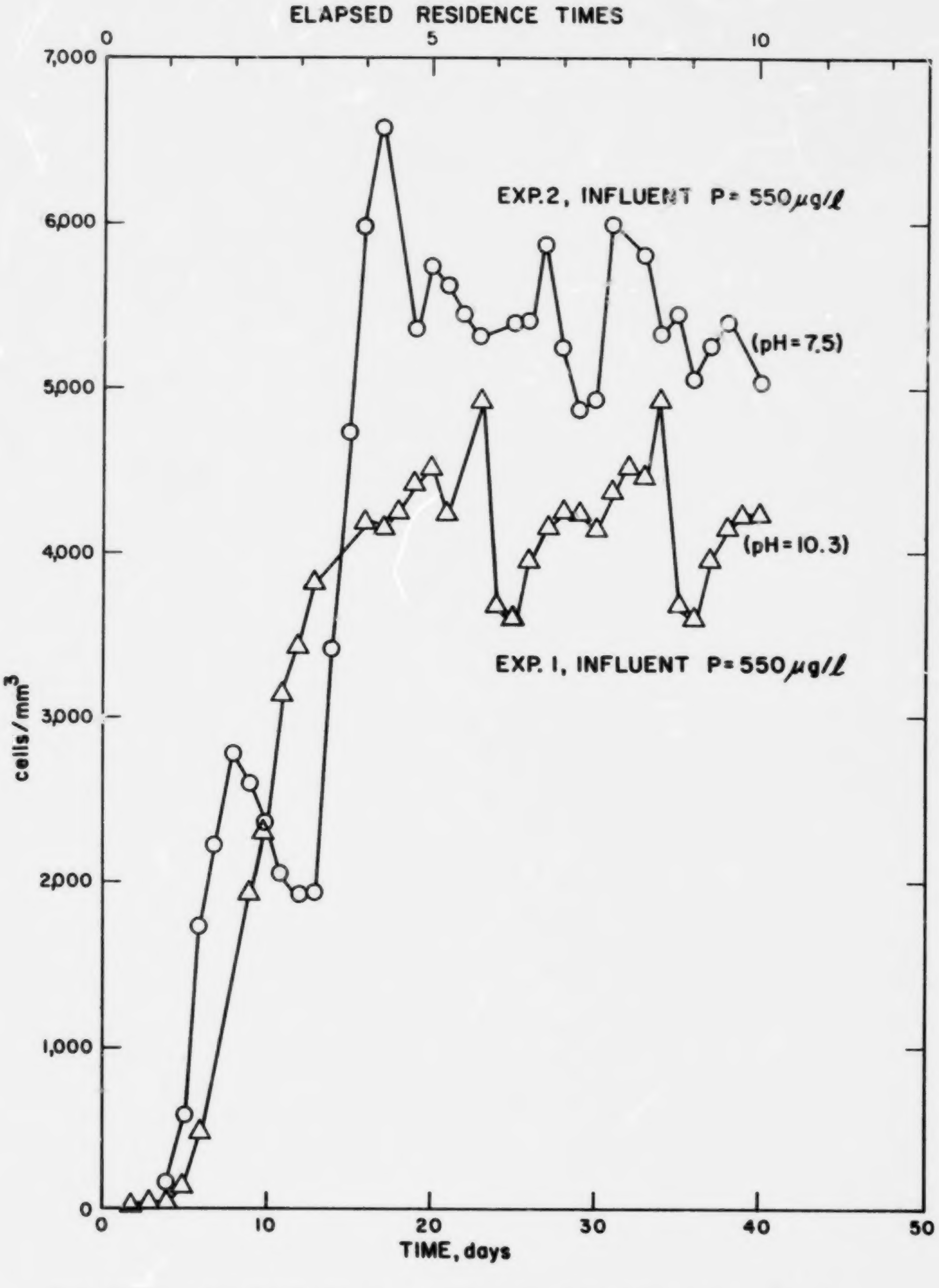

FIGURE 2. CHANGES IN CELL NUMBER OF *Selenastrum capricornutum* AS STEADY STATE IS APPROACHED UNDER CONDITIONS WHERE PHOSPHORUS AND CO_2-PHOSPHORUS ARE LIMITING

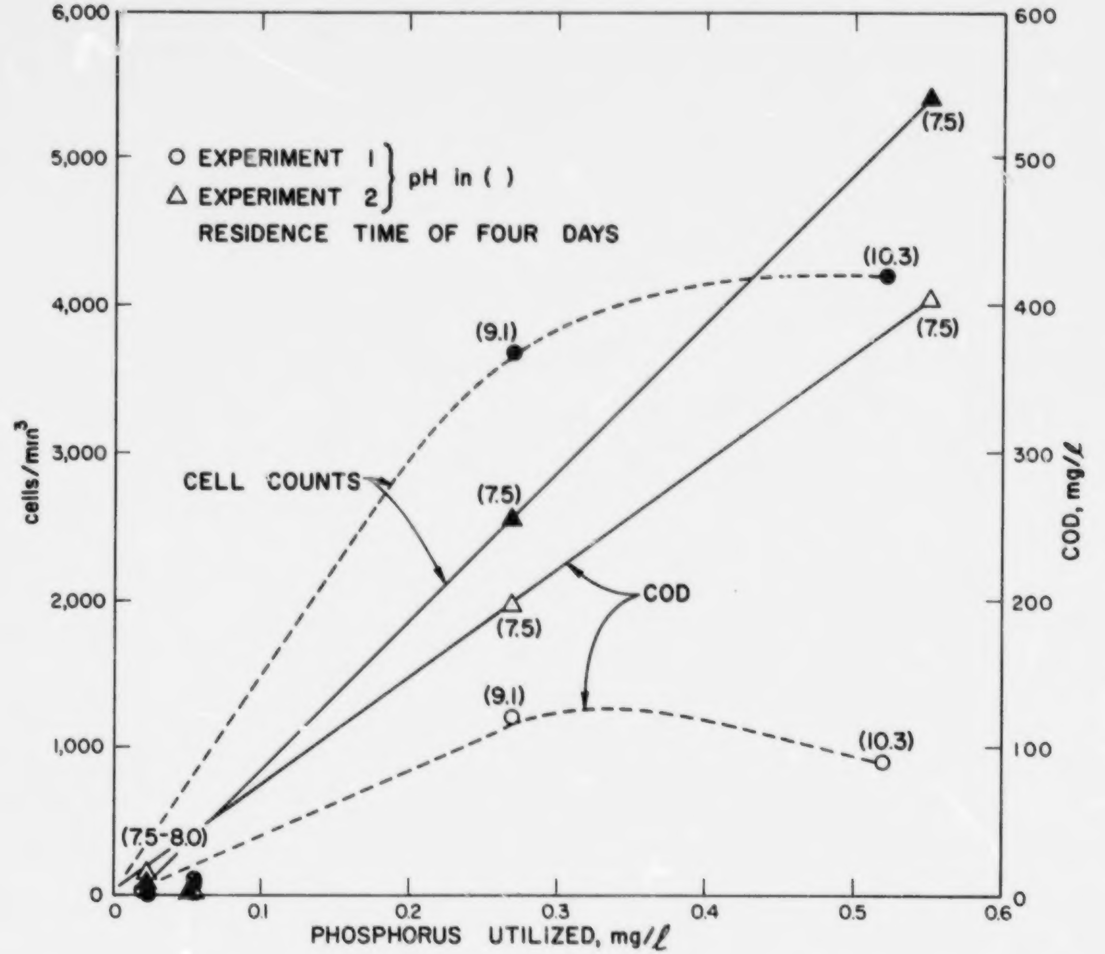

FIGURE 3. STEADY STATE YIELD RELATIONSHIPS BETWEEN CELLS AND PHOSPHORUS UTILIZED

of growth, interpretation is very difficult. Hence, it is very important to determine which factor is limiting the growth of the algal cells. The results shown in Figure 3 for Experiment 2 give a yield coefficient of 10^{10} cells produced per mg phosphorus utilized or about 10^{-10} mg of phosphorus utilized per cell which is in the range of cell content of phosphorus reported for other algal cells (summarized in Kuhl 1962). The corresponding relation between COD and phosphorus utilization was 725 mg COD produced per mg phosphorus utilized.

Assay Using Natural Algal Populations

To eliminate loss of nutrients caused by filtration and to make some comparison with experiments using test algae, a series of chemostat experiments were made on unfiltered samples at the Lake Tahoe Area Council Laboratory.* Results were obtained for chemostat analysis of the growth of natural populations of algae in raw sewage and sewage effluent (primary, secondary, and tertiary) samples from the South Tahoe PUD Water Reclamation Plant (January 18, 1969) and were diluted to 10 percent in unfiltered Lake Tahoe water. The experiments were carried out in cylindrical reactors mixed by aeration through the bottom (see Aleti 1969). Incident light intensity was about 2700 lux (250 ft-candles) and temperature was maintained at 20°C. A dry weight measurement (suspended solids) was used to estimate biomass. Results of biomass determinations on the chemostat effluent for the secondary and tertiary chemostats are shown in Figure 4.

Inspection of Figure 4 indicates a pattern which is similar to that obtained in Figure 2, an increase to a high value and then a falling off to a lower level which is maintained at steady state. Because the raw sewage and primary effluent reactors were similar to the secondary, they are not shown. Although the influent concentrations of nitrogen were relatively similar (16-18 mg/ℓ) for the two reactors shown, the tertiary treatment process represented a removal of about 98% of the orthophosphate. Hence, it was considered that the phosphorus or something removed with it probably limited growth in the tertiary reactor. For all the reactors, nearly complete utilization of phosphorus occurred but not nitrogen. Considerable nitrogen was in solution indicating that some other factor (light, phosphorus) was limiting.

In general the succession of algae in the reactors was as follows: Chlorella sp. was the first to appear in significant numbers and they increased to a maximum concentration and remained at that level throughout the experiment. Pennate diatoms increased slowly and then decreased as increasing numbers of blue-green algae, principally Anabaena sp., appeared. The final, steady state population was composed primarily of Anabaena. No change in suspended solids was observed to correlate with the appearance of the blue-greens.

Although an apparent steady state existed, it was only illusory. At the termination of the experiment the reactors were scraped to remove wall growth (diatoms and blue-greens), mixed well, and analyzed again. The weight of the cells was 3-30 times greater than determined in the effluent samples. As a result, an attempt to correlate the algal biomass in the reactors with the nutrient utilized was not very successful (Figure 5). However, the suspended solids concentration

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*This work and the results reported in the following section were done on the FWPCA Demonstration Grant No. WPD-48-01 (R1) and with the technical help of Peter Cowan and Mrs. Florence Kupka.

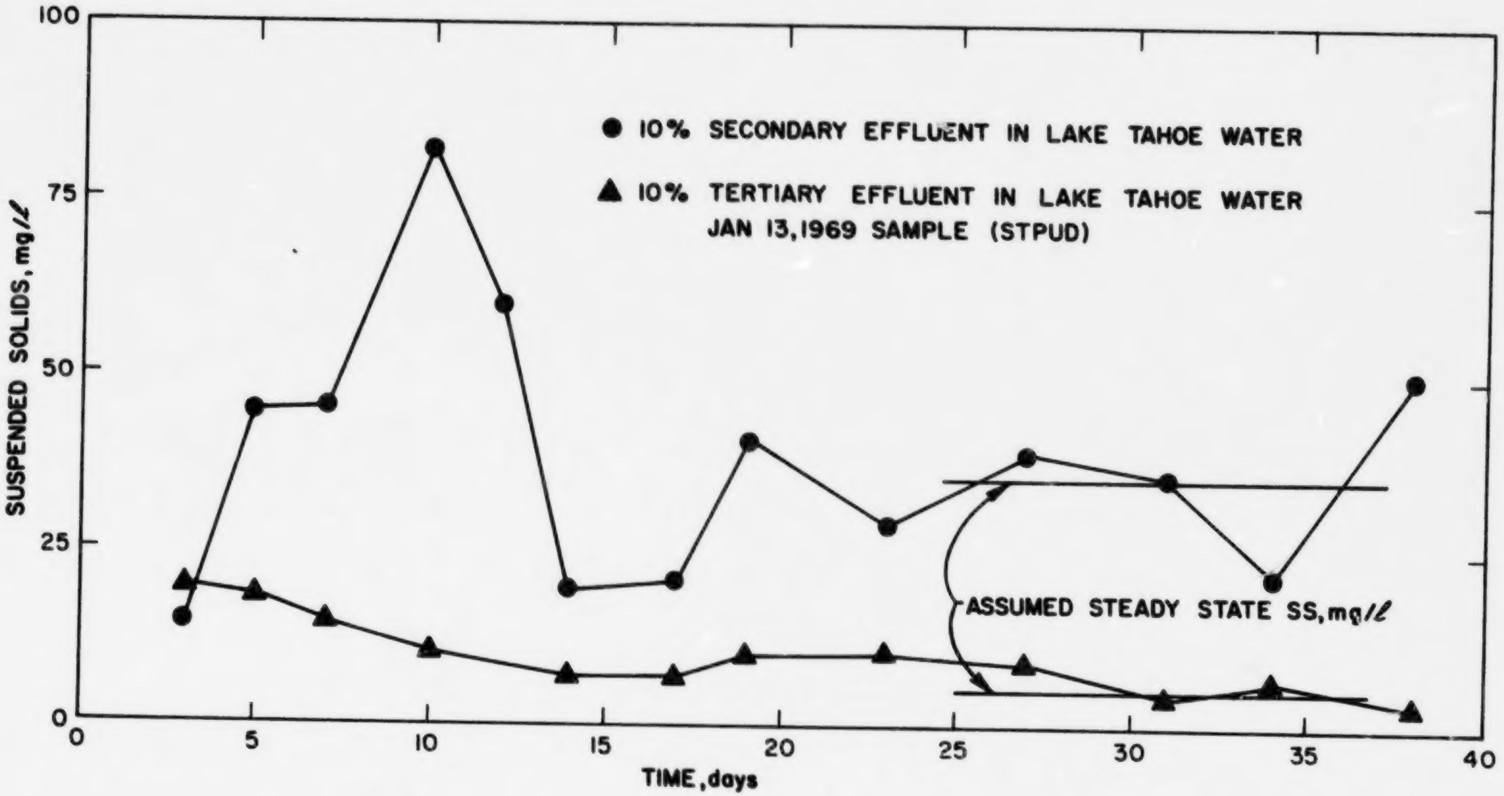

FIGURE 4. CHEMOSTAT ASSAYS WITH INDIGENOUS ORGANISMS OF SEWAGE EFFLUENTS DILUTED IN LAKE TAHOE WATER

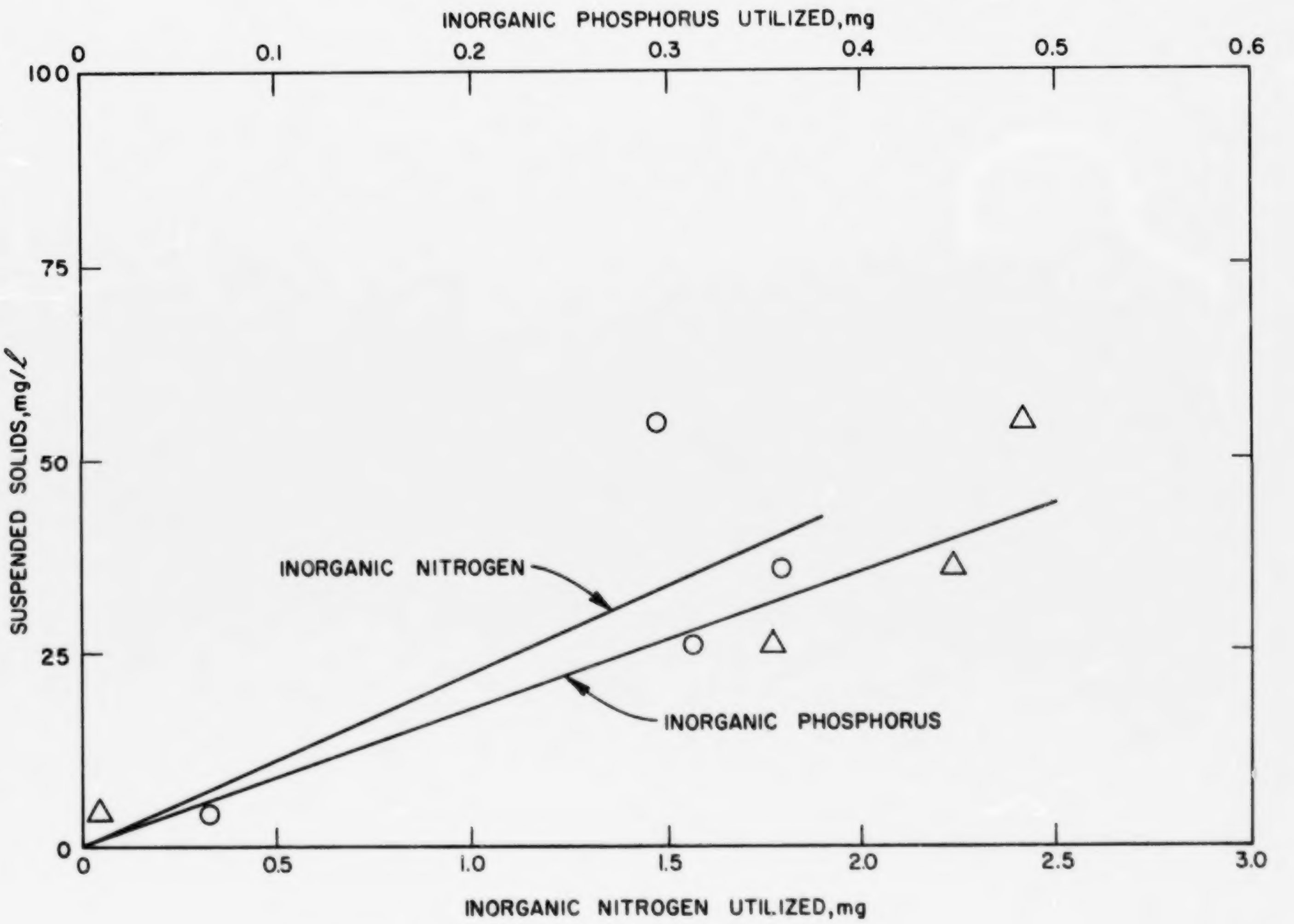

FIGURE 5. YIELD RELATIONSHIP OF BIOMASS OF INDIGENOUS ORGANISMS WITH NITROGEN AND PHOSPHORUS UTILIZED

obtained in the reactor receiving secondary effluent was a factor of 3 greater than had been obtained with the usual seed alga (*Selenastrum gracile*, Reinsch) used for bioassays (Aleti 1969). This indicated that the algae making up the so-called natural populations were better able to utilize the nutrients present in the secondary sample. The biomass concentrations obtained from the tertiary reactors were roughly the same for seeded and natural algae populations indicating that perhaps the blue-green algae were better able to grow than *Selenastrum* at the higher nutrient concentrations in the secondary effluents under the culturing conditions.

Washout of Seeded Populations

In the assay of low nutrient waters the algal population will grow only at a slow rate. Therefore extremely long residence times are required to obtain a steady state. Even at low residence times the cells may wash out of the system or be at such low concentrations that it is difficult to detect their presence. This phenomenon is illustrated in a bioassay at the LTAC Laboratory of a creek water sample collected December 3, 1968 and shown in Figure 6. Initially, growth occurs as the cells utilize the available nutrients. However, cell division ceases by day 5 and the cell number decreases according to the theoretical hydraulic washout curve. However, the suspended solids concentration decreases at a slower rate. Cell number follows the theoretical particle washout according to theory since probability governs the removal of individual particles (Danckwerts 1954). Apparently the remaining cells are still growing by increase in mass but cell division is not occurring. This could occur in the case of nitrogen or phosphorus limitation and probably also for other nutrients as it has been observed that carbohydrate buildup occurs when these nutrients are limiting (e.g., Kuhl 1968). In an assay of a water sample such as this, the rate of loss of cells from the system would be sufficient justification for considering that a steady state would not be reached, whereas the suspended solids data might be misleading.

CONCLUSIONS AND RECOMMENDATIONS

The results described above indicate three problem areas which have special application to chemostat bioassays. These same problems have reference to other bioassay techniques and it is possible that the reason they can be delineated so clearly is due to the sensitivity of continuous-flow methods.

Further research needs to be done in regard to multiple limiting factors and the identification of limiting factors, and the use of natural algal seeds for analyzing biostimulatory response. There are formidable difficulties in obtaining interpretable steady state measurements under conditions where several different factors control growth. More research on the relationships of extrinsic factors to biostimulatory response is especially needed to make certain that variation in light, temperature, or CO_2 will have no significant effect on the results. The use of natural algal seed populations in chemostats is not acceptable unless it is possible to insure that adequate mixing is achieved through wall scraping or perhaps specific inhibition of growth of those groups which become attached. For example, increased turbulence and selection by temperature or light intensity may allow one to obtain completely mixed systems.

The problems of washout are an area of research where nutrient additions should be made to determine their effect, e.g., as done above in the addition of sewage nutrients to Lake Tahoe water. However, difficulties in assaying directly

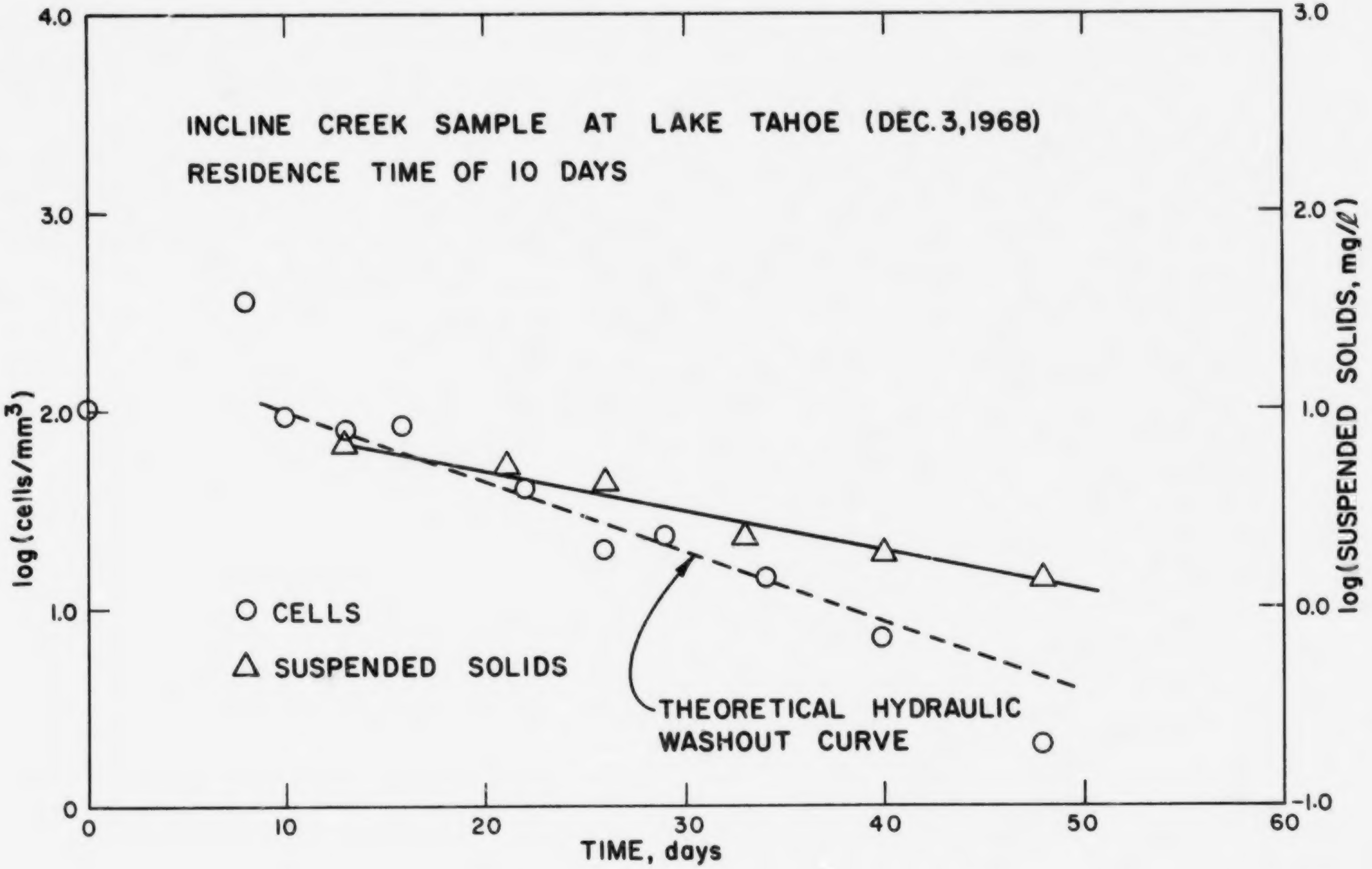

FIGURE 6. CHEMOSTAT ASSAY WITH *Selenastrum gracile* OF LOW-NUTRIENT WATER AND THE WASHOUT OF CELLS

the low nutrient concentrations involved would probably indicate against the routine use of chemostats for assaying low nutrient waters.

Two additional lines of research which have only been alluded to above must be approached to aid in determining biostimulatory response. More research with synthetic media should be developed so that specific limiting nutrients can be evaluated in terms of their individual kinetic parameters. Also more analysis of transient growth and the biomass and nutrient concentration profiles should be performed in order to evaluate kinetic parameters in terms of their transient characteristics. As a working hypothesis, it is very likely that for any particular small drop of water in a natural ecosystem it is in transient stages as opposed to steady state. Chemostats are useful tools for such studies because the algal growth system is dynamic yet is maintained at an average constant value.

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BATCH ASSAYS FOR DETERMINATION OF ALGAL GROWTH POTENTIAL

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The use of algal assay procedures to systematically determine the algal growth potential (AGP) of wastes or natural waters is relatively new and current investigations of this method of water quality appraisal have become international in scope. This breadth of investigative interest is to a great extent due to the enlightened leadership of the Federal Water Pollution Control Administration as embodied in the National Eutrophication Research Program (NERP) of the Pacific Northwest Laboratory and its director, Dr. A. F. Bartsch. The technique is now rapidly moving from a procedure used differently in every laboratory into a standard method. A basis for such standardization is the Provisional Algal Assay Procedures (PAAP) (1969) established by NERP following consultation with a national technical advisory board of sanitary engineers and biologists. Eventually the PAAP will become standardized to the AAP or similar title and will become part of the American Public Health Association standard methods. A procedure similar to PAAP is also under consideration by the American Society for Testing and Materials.

A glance at the history of laboratory algal cultures may be of some general interest. According to Pringsheim (1949), the first intentional algal cultures were grown by Famintzin and reported in 1871. However, as early as 1774 Joseph Priestly, in his pioneering work with electricity which led to the discovery of oxygen, often noted that the water in his laboratory flasks "turned green." (Retrospectively we may now feel that this sheds some doubts on the quality of his water, if not of his work.) Since at least 1880, it has been ordinary practice for botanists to cultivate algae in the laboratory in glass flasks. Pringsheim credits himself in 1912 as the first to use soil extracts to determine their nutritive needs, and credits Beijerink in 1890 as the first to use gelatin, a technique which ultimately led to pure cultures. Such cultures were prepared by placing a pinch of garden soil in water and inoculating it with some green material from a pond or aquarium and then adding a variety of enrichments, viz., a "bit of cheese," a "pea," etc., and incubating it on a "north window ledge." Pringsheim's enrichment techniques to isolate pure cultures of algae are used to this day although garden soil extracts are often replaced by unsterile sea water, dilute sewage, or soil extracts from a special locality. Often soil infusion must be used to cultivate algae demanding unknown growth factors.

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Controlled organic infusions for algae perhaps began with Pringsheim's pea-rice infusion for Euglena (1/2 split pea and 3 grains of rice boiled in 1 liter of tap water) but the general trend for cultivating algae was away from unknown, unsterile solutions toward such scientific preparations as Knops' or Gorham's media. Algae has been used by nutritionists to assay foods for certain vitamins and occasionally soil scientists attempted to use algae grown on infusions of specific soils to measure and appraise the availability of fertility in the soils, but such fertility tests are more often done with potted plants. By 1945, glass flasks containing liquid suspensions of algae had been used to demonstrate lake fertility (Lackey 1945) and later they were common in sanitary chemistry laboratories to demonstrate taste and odor relationships (Rohlich 1949). During the 1950's such cultures were used for appraisal of algacides and river fertility (Palmer 1960) to examine trace nutrient requirements of algae (Eyster 1969) and to appraise various wastes for their potential as a nutrient for the cultivation of algae (Oswald 1960). Such work naturally led to the concept of aquatic fertility as a conservative parameter subject to appraisal in bioassays (Oswald 1966, 1967; Fitzgerald 1969). Our primary concern today, however, is not with ancestor worship but rather with a description and discussion of bioassay techniques which we hope will ultimately lead to better methods of evaluating and studying algae cultures, eutrophication, and biostimulation (Middlebrooks 1969).

My assigned purpose today is to define, describe, and perhaps to defend the batch method for algal assay. As in all new endeavors, we are immediately confronted with a series of name changes. For example, our workshop program entitles our presentation Batch Test, whereas described in the Provisional Algal Assay Procedures, the batch test is called the Bottle Test! Batch or bottle, this test involves a procedure described briefly as follows:

> "In the bottle test, candidate test algae are added to the sample and (following incubation under specified conditions) growth is determined by assaying the algal crop on a daily schedule. The immediate test result is a measure of the algal mass at any given time. From this a provisional growth rate can be derived."

The first part of the discussion deals with the section (pages 7-30) of Provisional Algal Assay Procedures (1969) concerned with the "Bottle Test."

My remarks will be based primarily on my own experience in the conduct of tests of this sort — experience which doubtlessly has led to some erroneous views and biases which will no doubt be changed by newer workers untrammeled by experience and unconfused by the "facts." In the second part of this discussion I will say a few words intended to contrast the batch or bottle tests with other procedures such as the continuous flow chemostat and the *in situ* test.

The general requirements for a useful algal assay procedure are that it be simple, inexpensive, reproducible, universal, and applicable to practice. I shall endeavor to relate these parameters to the batch test as they become apparent.

Beginning with a brief description of the batch technique, it is essentially one of applying a specific algae to a specific sample under specific growth conditions and determining either the progress of growth or total growth in a specified time. While I feel that progressive growth studies are significant, ultimate growth is a most significant factor for quality evaluations since it reveals the quantity of algae which could grow in a given water should no factor other than nutrient limit growth. It essentially establishes a ceiling beyond which algae growth cannot go regardless of environment and thus is an excellent parameter for specification, regulation, standardization, and so on. Thus batch appraisal meets the standard of applicability.

2.1* In the detailed description set forth in PAAP, use of rigorous temperature control is specified. According to our experience, use of a constant temperature room or similar mechanisms for temperature control at 24°C ± 1°C may not be absolutely essential. As is shown in Figure 1, according to our study of the influence of temperature on Chlorella, temperatures between 18°C and 25°C should give similar results. However, I have no objection to controlled temperature studies, should these be attainable.

2.2 and 4.62 Illumination with a standardized lamp of low intensity is essential. The "cool white" fluorescent lamp recommended is adequate; however, more attention to intensity is required. It is likely that the recommended intensity of 400 ft-c will be excessive for new cultures of Selenastrum or cultures in weak substrate, but will be satisfactory for old cultures or strong substrate. The use of screens may be too complex or awkward, particularly when intensity control can also be attained by increasing the distance from lamp to culture.

2.6 and 4.63 This recommendation calls for a shaker capable of 80 oscillations per minute. This quantity of shaking is far in excess of any requirement for mixing or gas exchange and does little to aid in avoiding the clumping or encrusting of cells on the flask sides. Our apparatus (see Figure 2) does not provide for shaking. Daily swirling plus mechanical cleansing of the flask sides with a plastic brush, which remains with the culture throughout the test to avoid contamination, has been found adequate for all but the fastest growing algae.

2.9 This section of the PAAP has a typographical error, and should read Erlenmeyer flasks 25 to 1000 mℓ rather than 25 to 100 mℓ. Use of Erlenmeyer flasks as culture vessels is ideal. There is a close relationship between volume of flasks and volume of culture with respect to influences of required shaking. Our best success has been with a 100-mℓ volume of culture in a 250-mℓ Erlenmeyer flask. For most applications in such a flask, daily shaking is adequate.

2.10 The application of covers to the culture vessels is recommended in PAAP. We have used open culture vessels which improves the opportunity for gas exchange and helps to obviate the need for continuous shaking. A properly constructed light box prevents the intrusion of dust and insects into open flasks and makeup water can be applied for evaporation control.

4.3 With regard to species, our experience has shown that regardless of the species of test organisms, such organisms should be used only after they have been adapted to the specific water under study. This usually can be accomplished by culturing the test organism in the waste for several weeks. As an example of adaptation we found in our studies with potato wastes (Middlebrooks 1969) that adapted cells initially grew 10 times as fast as unadapted cells, thus reaching their ultimate growth in a shorter time.

Unfortunately we have not had an opportunity to study the growth characteristics of Selenastrum capricornutum, Anabaena flos-aquae, or Microcystis aeruginosa with the same thoroughness that we have Chlorella pyrenoidosa, Scenedesmus obliquus, and Euglena gracilis.

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*Numbered paragraphs cited from PAAP (1969).

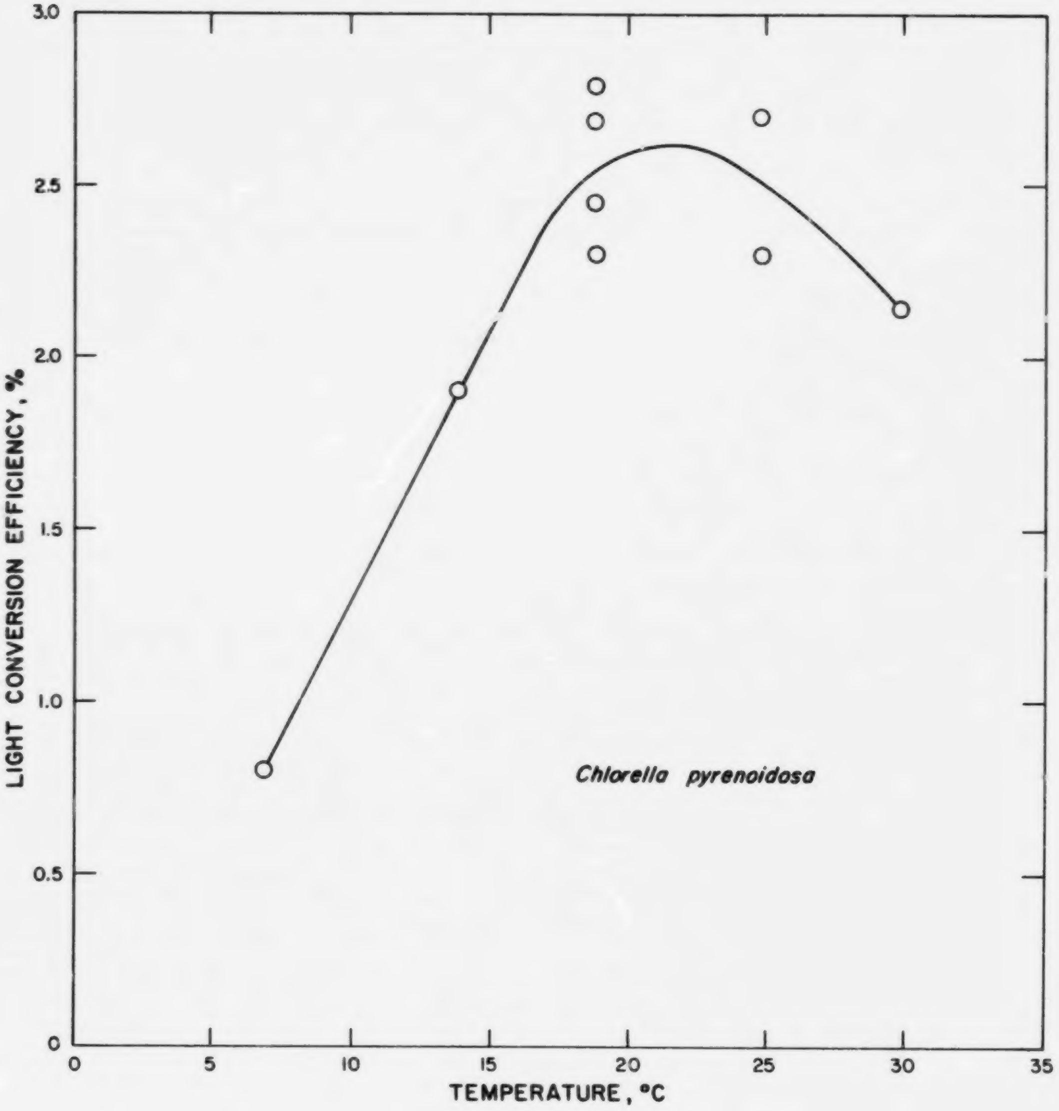
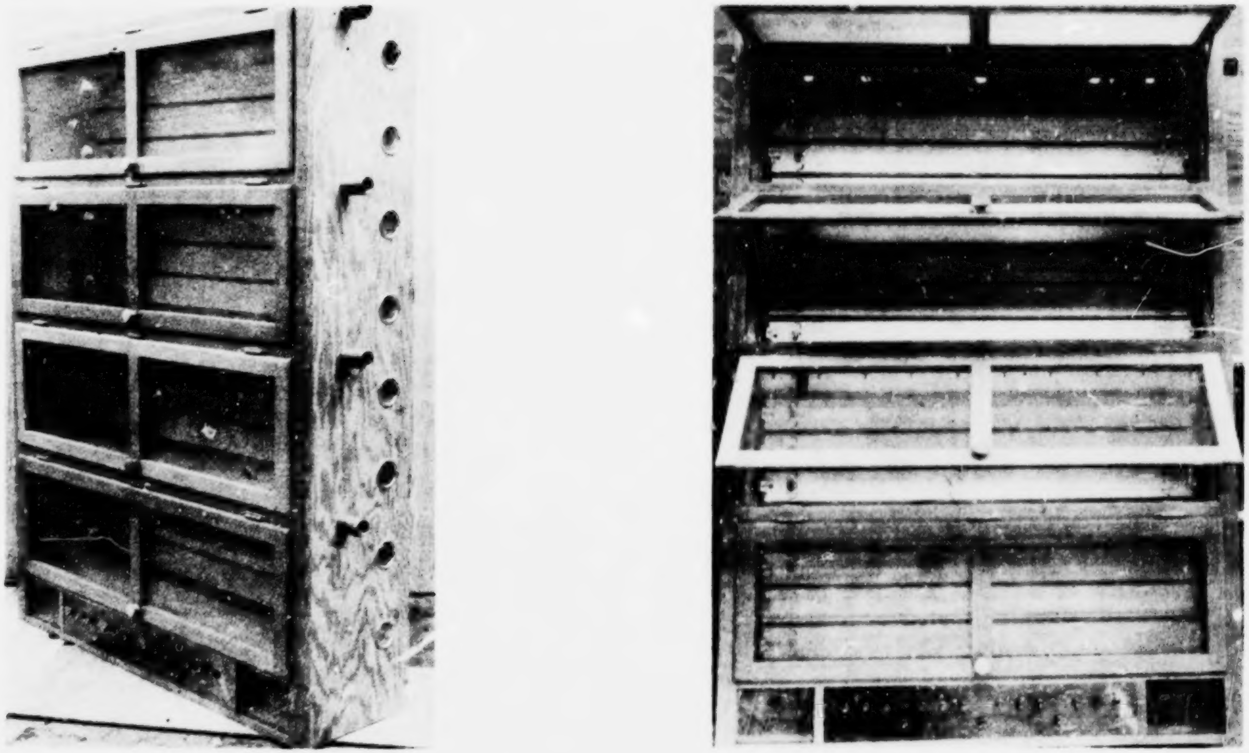


FIGURE I. INFLUENCE OF TEMPERATURE ON THE GROWTH OF *Chlorella pyrenoidosa*



A. BOX CLOSED

B. BOX OPEN

FIGURE 2. LIGHT BOX FOR INCUBATION OF BOTTLE ALGAL ASSAYS

4.4 With respect to PAAP, the use of several dilutions is recommended as one set of test flasks. As indicated in Figure 3, this is an extremely valuable technique since it will reveal toxicity or stimulating characteristics. Care must be exercised with the quality of the distilled water used.

Also in section 4.4, spiking with phosphorus and nitrogen is recommended. Fitzgerald (1969) has found this technique to be revealing with respect to limiting nutrients. As indicated in Figure 4, studies involving spiking with iron, as well as with nitrogen and phosphorus, will yield dramatic results.

In PAAP, four methods of determining growth are suggested: 1) gravimetric, 2) absorbance, 3) cell count, and 4) ^{14}C. It is my considered judgment that two additional techniques for evaluating growth should be studied. These I shall refer to as 5) fluorescence and 6) volumetric. Fluorescence has been studied by Bailey (1967) and its application will be discussed by Mr. Bain later. The volumetric technique (Oswald 1966) like fluorescence involves a procedure in which a sample is not sacrificed and therefore may be evaluated a number of times in order to follow the time course of its development: Used is the centrifuged packed volume determination. By centrifuging 100 mℓ of the incubated cultures at 500 times gravity for 10 min in a 100-mℓ Goetz pear-shaped centrifuge tube, the packed volume of algae can be observed in the graduated stem. This volume multiplied by 10 is the packed volume in mℓ/ℓ. The packed volume usually contains approximately 14 percent dry weight of algae; therefore, if the packed volume from 100 mℓ, in mℓ, is V, the approximate algal growth in dry mg/ℓ is 1,400 V ± 400. (See Figure 5.)

Certain Goetz tubes have a calibrated stem with maximum reading of 0.2 mℓ and a least reading of 0.01 mℓ; hence, an AGP of 10 to 15 mg/ℓ may be detected without resorting to concentration of the sample. For water having AGP's in excess of 200 to 300 mg/ℓ, a Goetz tube with maximum reading of 2 mℓ and minimum reading of 0.1 mℓ may be used, or the water may be subjected to several dilutions in order to obtain levels of AGP. Dilution, however, changes the osmotic pressure of the water as well as its nutrient level.

There is little question that the volumetric method described above has advantages over the cell-count method, particularly when a heterogenous culture of algae (rather than a single-species culture) is used. This is obvious when it is realized that if one takes the size of *Chlorella pyrenoidosa* as unity the size of *Scenedesmus obliquus* is 1.2, *Chlamydomonas* 6.9, *Euglena gracilis* 54, *Euglena viridis* 84, and a *Phacus* sp. 71.

In addition to variability among species, cell counts have the disadvantage of variation in cell size both with age and with the nutritional environment of a given species. Among algal species there is apparently no consistent way in which cell size varies with culture age. For example, *Euglena* cells tend to get larger, while *Chlorella* and *Scenedesmus* cells tend to get smaller. The opposite is the case with regard to the effect of nutrition. Nutrient starved *Euglena* tend to grow progressively smaller with decreasing nutrient while nutrient starved *Chlorella* tend to enlarge with decreasing nutrient. Thus in *Chlorella* division is nutrient-linked while in *Euglena* it is not. I have found no statements regarding the pleomorphic characteristics of the several recommended test organisms in PAAP but such information should no doubt be established.

Other problems exist which are directly involved with enumeration. For example, many algal species are dark-light regulated. In the case of *Chlorella*, it characteristically accumulates nutrients during the day and divides during the

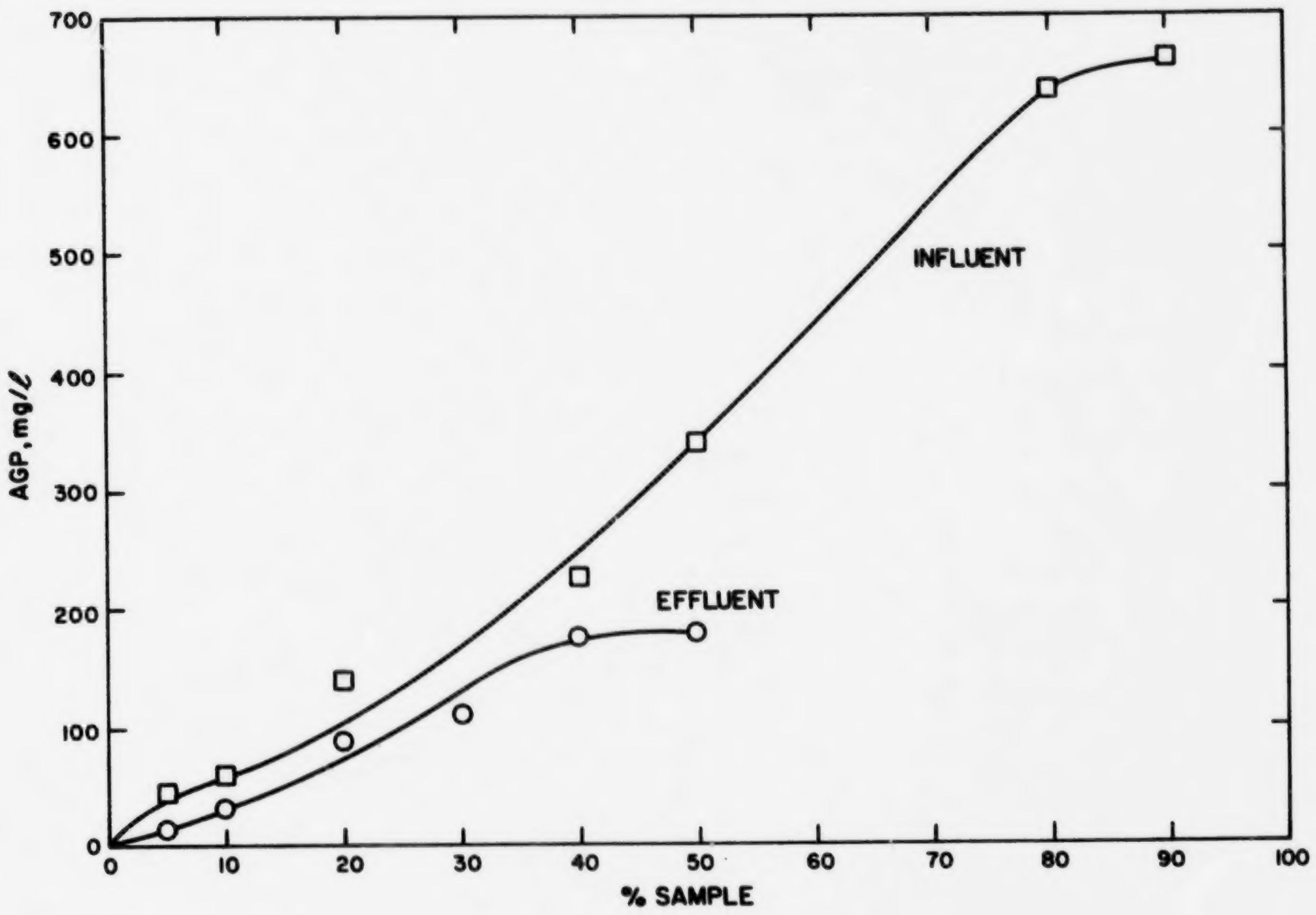


FIGURE 3. AGP OF HIGH-RATE ACTIVATED SLUDGE TREATMENT PLANT INFLUENT AND EFFLUENT

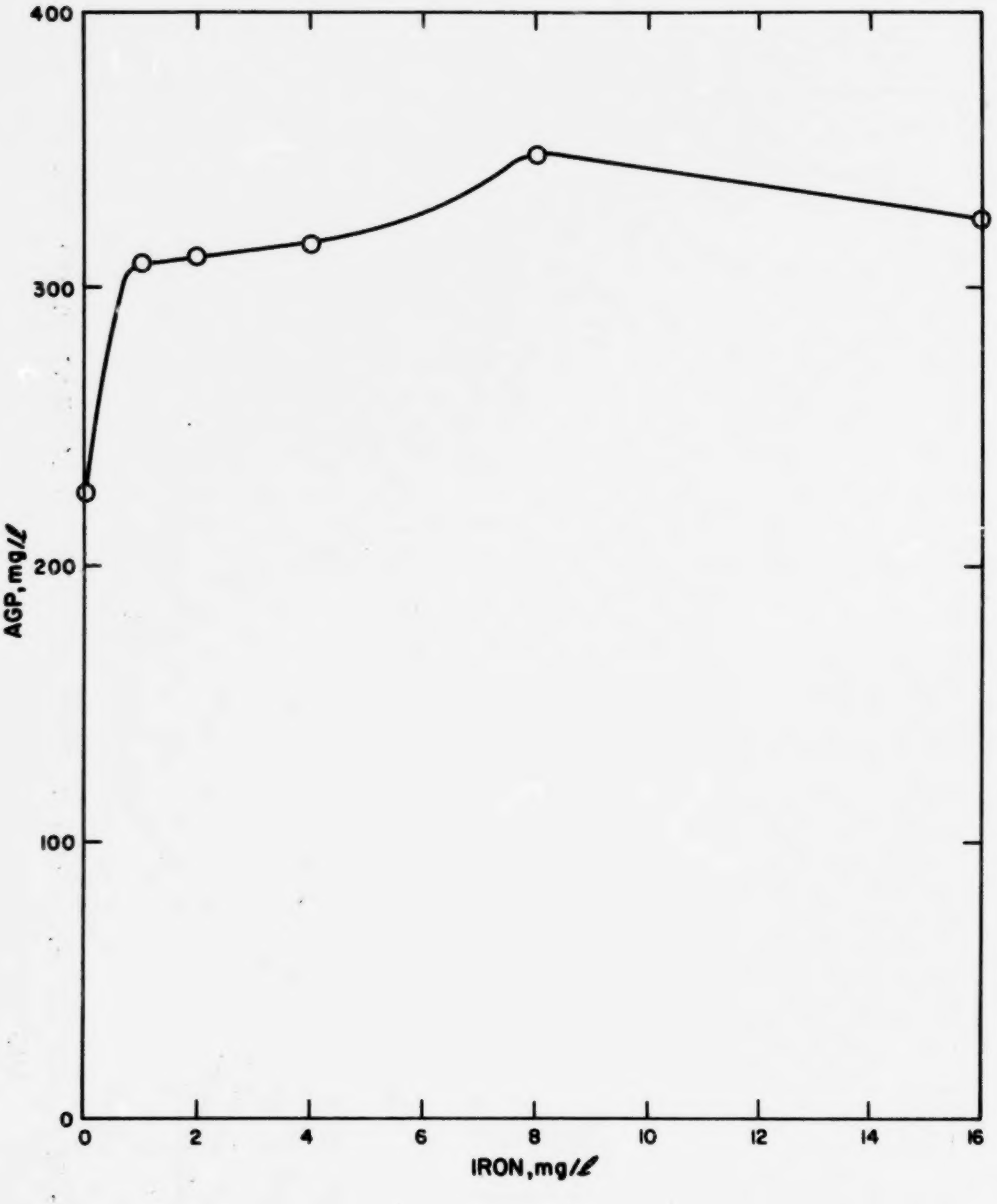


FIGURE 4. EFFECT ON AGP OF ADDING VARIOUS CONCENTRATIONS OF IRON IN 40% SAMPLE OF DOMESTIC SEWAGE (c.f. FIG. 3)

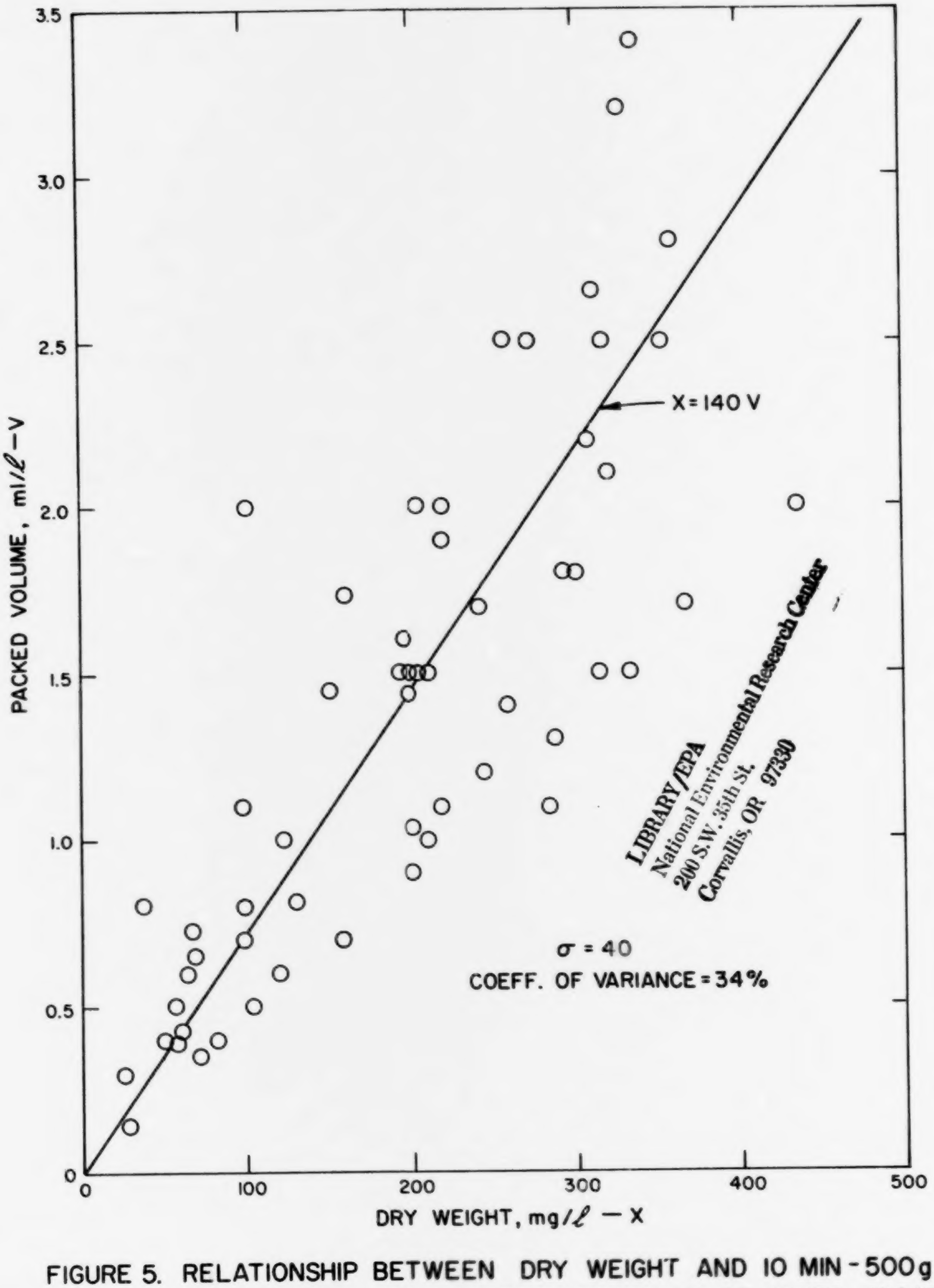


FIGURE 5. RELATIONSHIP BETWEEN DRY WEIGHT AND 10 MIN - 500g CENTRIFUGED VOLUME FOR CULTURES OF MIXED ALGAE

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night. It follows that unless counting were done at exactly the same time daily the number of cells found could easily differ by a factor of 10 during that day.

In addition to the real problem of culture variability, we have the technical problem of variations in cell enumeration with the haemocytometer. As shown in Figure 6, Hee (1954) studied this to some extent a number of years ago. In the case of Euglena, variation exceeds 30% when the number of cells counted in the haemocytometer grid is less than 30 whereas clumping occurs when the number of cells exceeds 100. As shown in Figure 7 in the case of Scenedesmus, a minimum of about 15% variation in count occurs when the number of cells counted in the 5 central squares exceeds 80. As evidenced in Figure 8 in the case of Chlorella, a minimum of about 10% variation occurs when the number of cells encountered in the 5 central squares exceeds 1000. This evidence is presented primarily to emphasize the degree of variability found in systematic cell count. Information similar to this should be made available with the test organisms recommended in PAAP.

Use of a Coulter Counter has been suggested as a remedy for the drudgery problem of evaluating a large group of cell numbers and for improving their reproducibility. The Coulter system has a disadvantage in that it is costly and thus may not be within economic reach of many laboratories.

Last but not least, a problem exists with regard to count variations among technicians. Although this is a real problem, it is one that is shared among all laboratory techniques.

In view of the ease with which actual dry weight may be determined using the millipore filter technique, or estimated by the packed volume technique, one might question the use of counts. Counts have the major advantage of being determinant at concentration far below those which are measurable gravimetrically. Fluorescence (Bailey 1967) is an excellent technique for early, simply determined results which does not involve destruction of the samples. However, results are only relative and therefore do not meet the gravimetric requirement of PAAP. Moreover, clumping of algae can interfere with the technique. Absorbance measurements are also virtually useless when clumping of the algae has occurred (which frequently is the case). The technique of radiocarbon measurements as set forth in PAAP seems needlessly complex, delicate, and subject to error in the hands of inexperienced personnel. A rational system could involve counts for AGP's from 0.1 to 10 mg/ℓ, fluorescence for AGP's 10 to 100 mg/ℓ, and gravimetry for AGP's 100 to 1000 mg/ℓ. A technique utilizing, say 15 flasks, 3 of which could be processed every two days, would permit gravimetric determinations of growth as a function of time.

The duration of a standard bottle test has not been determined and therefore is not stated in PAAP. Under the recommended continuous illumination required with a rapidly growing algae and with an adopted algae seed, it seems quite likely that an incubation period of 10 days should be adequate.

Our experience indicates that in cases in which a light-dark cycle is applied, much longer incubation periods may be required to reach a growth plateau. Our interpretation of this evidence is that due to the release of nutrients during the dark period, 30 or 40 days of 50% light, 50% dark may be required to be equivalent to 10 days of continuous illumination.

Concerning the condition of spiking cultures with added nutrients and CO_2, it is obvious that by adding one substance after another the AGP of any water can be

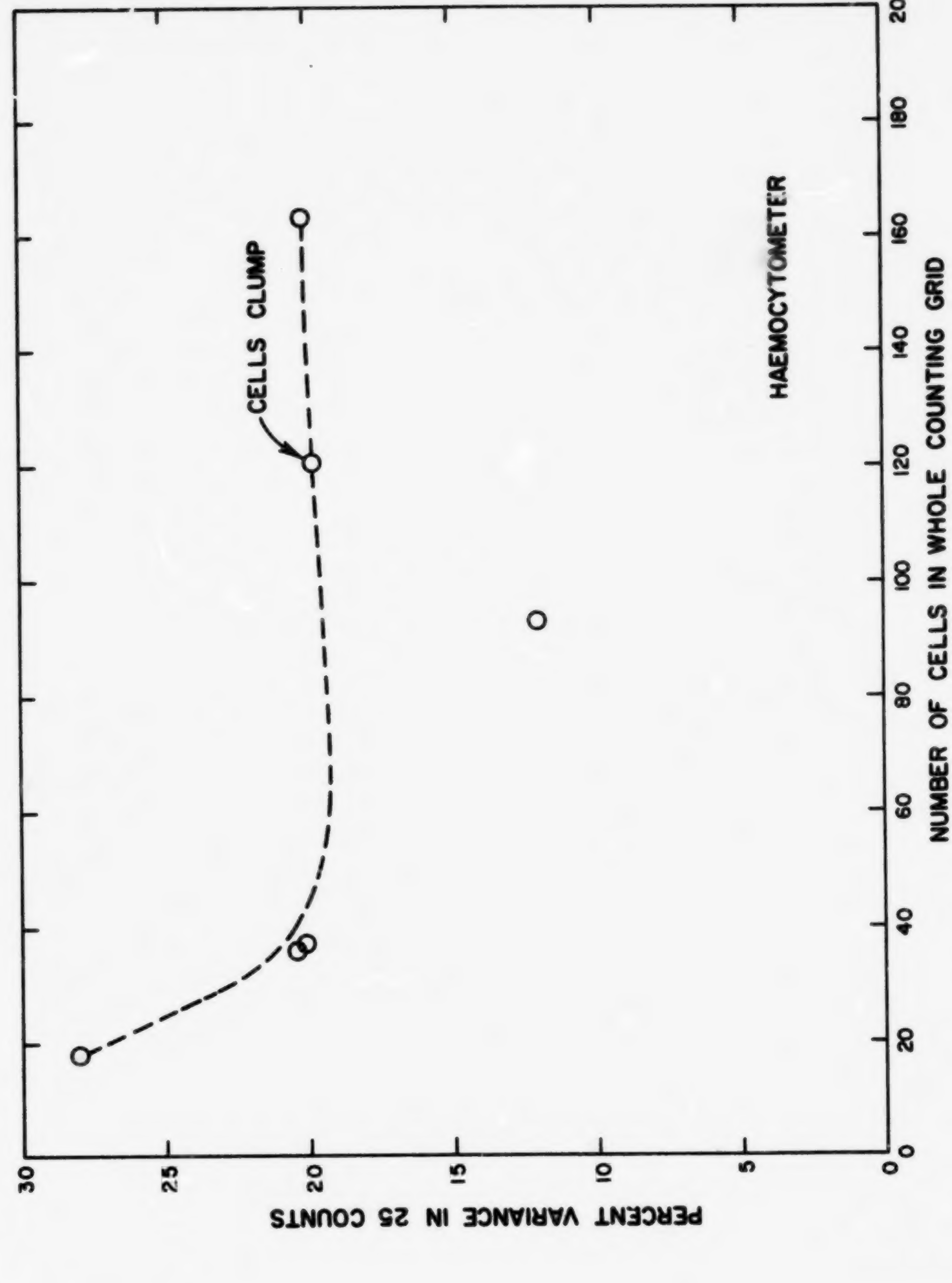

FIGURE 6. RELIABILITY OF *Euglena* COUNTS

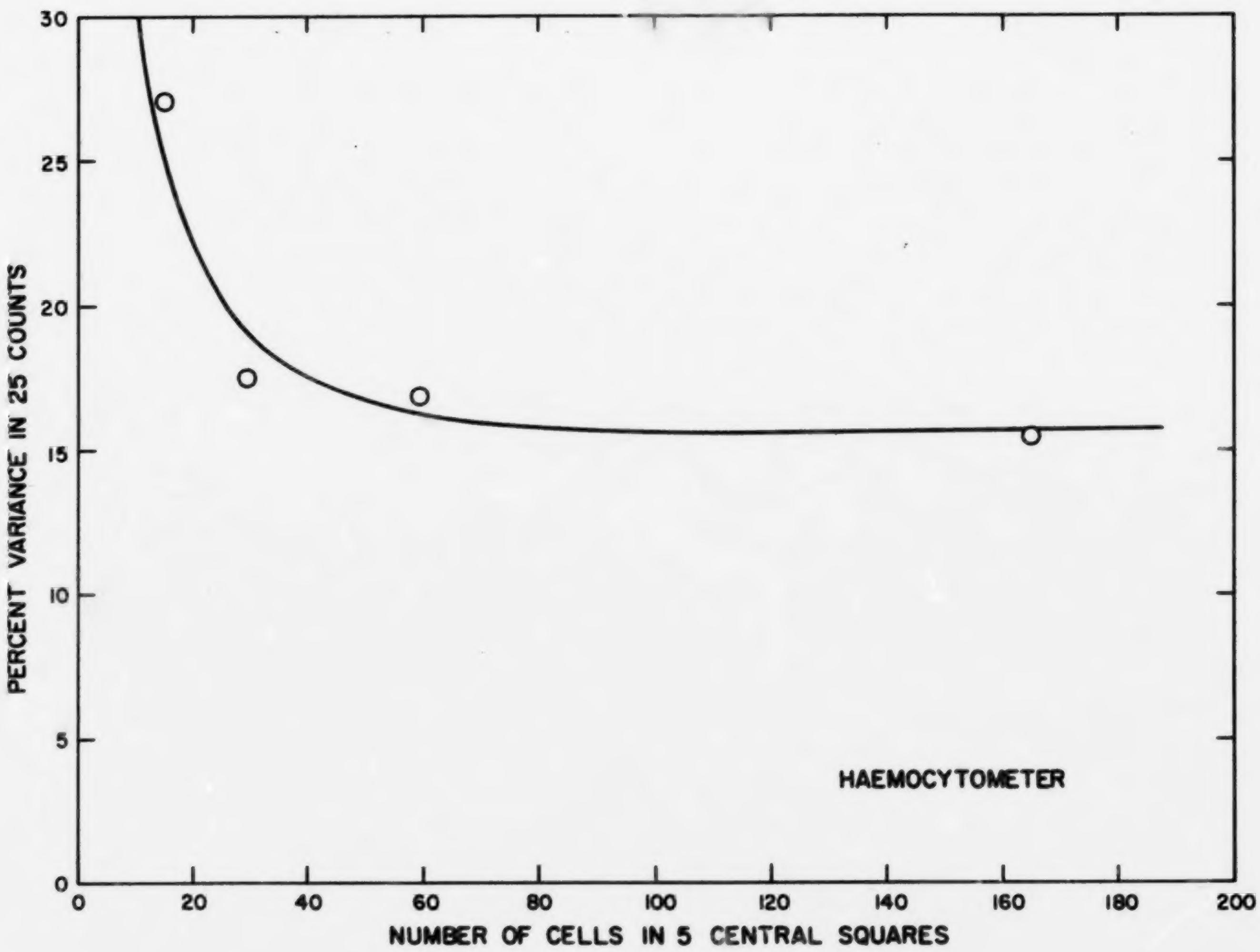

FIGURE 7. RELIABILITY OF *Scenedesmus* COUNTS

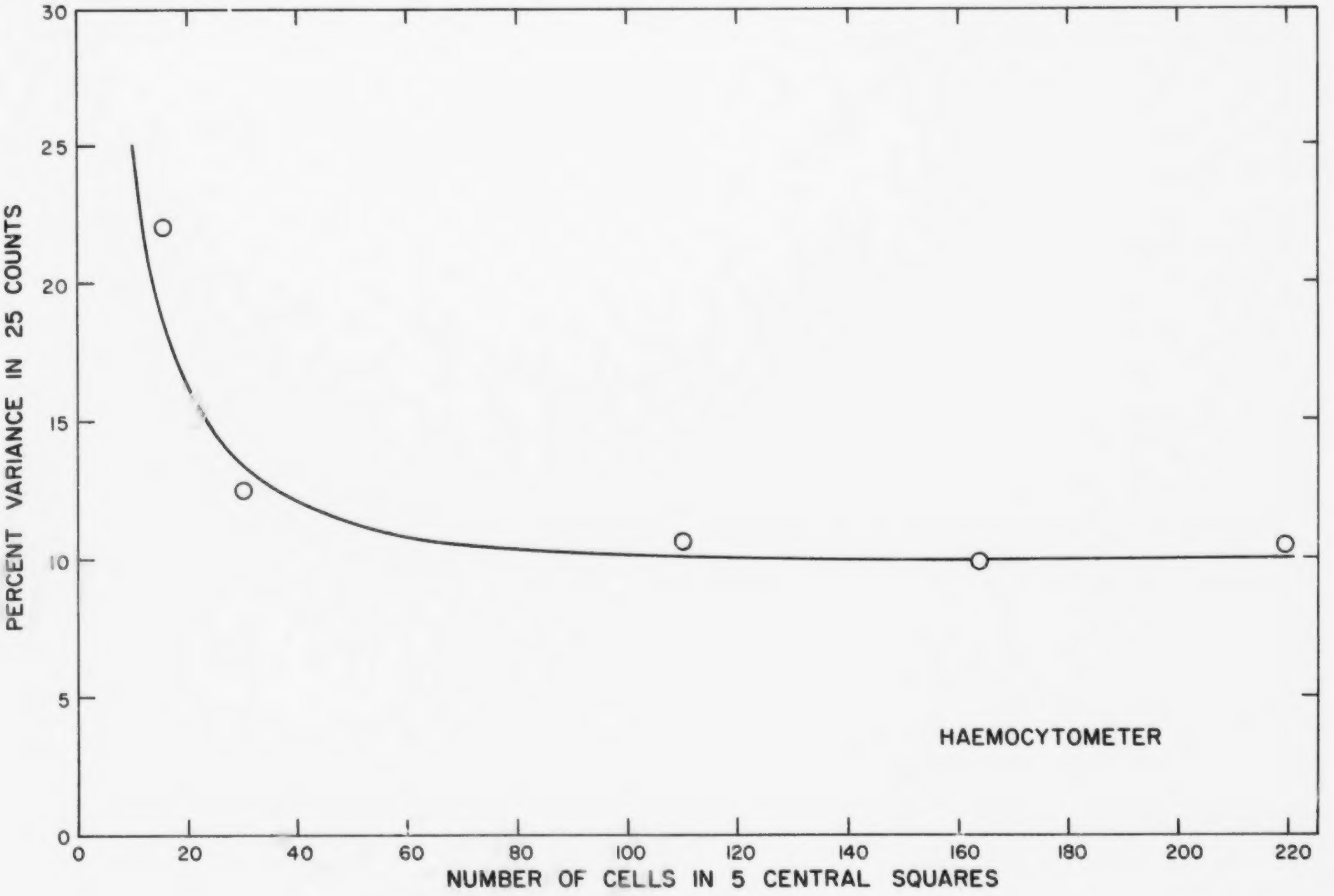

FIGURE 8. RELIABILITY OF *Chlorella* COUNTS

increased indefinitely. Spiking procedures are, of course, relevant when a search is being made for limiting factors (Fitzgerald 1969).

An area of great interest with respect to wastes and receiving waters is the not unexpected finding that the AGP of two waters, when mixed, does not equal but usually exceeds the computed AGP's of the two waters based on mixing formulas (Oswald 1968). This effect is termed synergism and merely reflects the fact that the number of nutritional factors which may be limiting to algal growth in several waters is reduced by addition of new waters. It is, of course, also possible that the addition of waters containing toxic substances may reduce the AGP of a mixture of several waters below that of one of the waters but not below that of all of the waters.

We turn now to consideration of the relative merits of the bottle test as compared to continuous flow chemostatic test or the in situ test. As a basis for discussion, it is worthwhile to reintroduce the stated objectives of the assay procedure as outlined in PAAP. These will be given as a statement followed by a short comment.

1. They should be so designed that technician-level personnel can do them.

   Having trained a number of inexperienced personnel to conduct the bottle tests, and having operated chemostats for a number of years, the bottle test seems to stand high above the chemostat in simplicity. There is no question that the chemostat requires trained scientific personnel to obtain results. I have no doubt that the in situ test also requires trained personnel.

2. Equipment and instrumentation requirements should be relatively modest and readily attained.

   There seems to be no real comparison between a simple light box incubator, Erlenmeyer flasks, and a millipore filter kit, as compared with the complexity of chemostats or the attainability of a stable place within a lake for in situ tests.

3. The procedures should be so standardized that results are acceptably reproduced.

   Here, theoretically, there should be no difference in the several tests. However, experience has shown that chemostats are subject to the typical problems of complex mechanical systems, namely breakdowns, and in addition they run out of materials unexpectedly, particularly on weekends. General reproducibility does not seem to be a relevant criterion in the case of in situ assays.

4. Geographic location should not affect the test results.

   This criterion is difficult to resolve with respect to in situ studies but is equivalent in the other two.

5. The results can be applied with judgment to field conditions.

   All candidate tests appear capable of meeting this criterion.

SUMMARY

Bottle tests similar to those now recommended for the algal assays have been employed by workers for almost 20 years in appraisals of algal growth potentials, trace mineral analyses, taste and odor studies, and toxicity studies. Because of its success in those investigations, there is no reason to doubt that the bottle technique will find widespread use in nutrient assay. What the bottle test lacks in elegance it has in simplicity, flexibility, versatility, reliability, and applicability. Certain characteristics of the batch or bottle test, as currently recommended in PAAP, require simplification and elaboration. Vigorous shaking and rigid temperature control, and the use of accessory gases seem relatively needless and should perhaps be evaluated along with simplified incubation techniques to determine if they are truly essential. The various parameters of growth for PAAP should be evaluated in terms of the level of algal growth for which they would be most useful. Thus, counts would be useful for the lowest growth levels and gravimetry for the highest growth levels. Several of the candidate techniques for growth progress evaluation seem needlessly complex. A broad program of PAAP evaluation is justified to avoid the pitfalls of preconception or dedication to useless techniques. The rewards in improved water quality resulting from a well-conceived standardized test or tests for algal growth potential will far offset the minor costs of the test appraisal program now under way.

ACKNOWLEDGMENT

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ALGAL GROWTH ASSESSMENTS BY FLUORESCENCE TECHNIQUES

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Bioassay procedures for assessing algal growth have recently been proposed by the Joint Industry-Government Task Force on Eutrophication (1969). These provisional algal assay procedures (PAAP) include static (batch), continuous flow (chemostat), and in situ tests. Algal crops are measured by a variety of techniques including cell counts, absorbance, gravimetric, and carbon-14. Static or batch type procedures suggested are similar in principle to earlier tests performed by Skulberg (1966) and Oswald (1966) but more complex in the degree of procedural detail suggested.

Chlorophyll fluorescence may be appropriate as a means of algal response measurement in the Provisional Algal Assay Procedure. Such an assay procedure has been employed by the author and his colleagues for approximately three years. During this period, waters from many different rivers, lakes, and estuaries have been tested for relative algal response and for reactions to nutrient additions and dilutions.

Chlorophyll pigments will fluoresce if excited with ultraviolet light. This phenomenon is the basis for fluorometry techniques applied to chlorophyll standing crop measurements (Yentsch and Menzel 1963; Holm-Hansen et al. 1965). Solvent extraction procedures using acetone solutions are more commonly employed although direct readings of chlorophyll fluorescence of phytoplankton are also possible where cultures are reasonably dense (Lorenzen 1966). Readings may be taken with discrete samples or by continuous flow through a fluorometer equipped with suitable filters. Filters recommended for chlorophyll a response include Wratten blue 47B or Corning CS-5-60 for excitation and Wratten red 25 or Corning CS-2-60 for emission (Yentsch and Menzel 1963; Holm-Hansen et al. 1965). Fluorescence may be related to chlorophyll a through calibration with spectrophotometric techniques or by chromatography.

The procedure used by the author includes laboratory or field incubation of vessels containing natural waters with native algal flora with or without specific nutrient inocula. Incubations are generally for two weeks using either 12-hour light – 12-hour dark cycles or with continuous light in laboratory tests. In situ experiments have generally been conducted in floating amber glass bottles or translucent containers to prevent excessive solar illumination. Subsamples are taken for chlorophyll fluorescence every day or two depending on the experiment. Subsample volumes may be 20-50 mℓ if acetone extraction procedures are used; where algal crops are more dense, direct readings permit a smaller subsample volume which in some cases can be returned to the incubation vessel. The chlorophyll extraction procedure used is covered in some detail in the appendix.

The laboratory incubator currently used consists of a walk-in meat locker held at approximately 20°C. Lighting is by fluorescent tubes (Durotest Optima Series) in a blower-ventilated box painted white for maximum reflectance. Tubes are mounted below a clear glass plate which supports the incubation vessels; light intensity at the plate surface is generally held between 500 and 1000 foot-candles; lower intensities are achievable through filters or screens. See Figure 1 for photograph of incubator currently in use for laboratory tests.

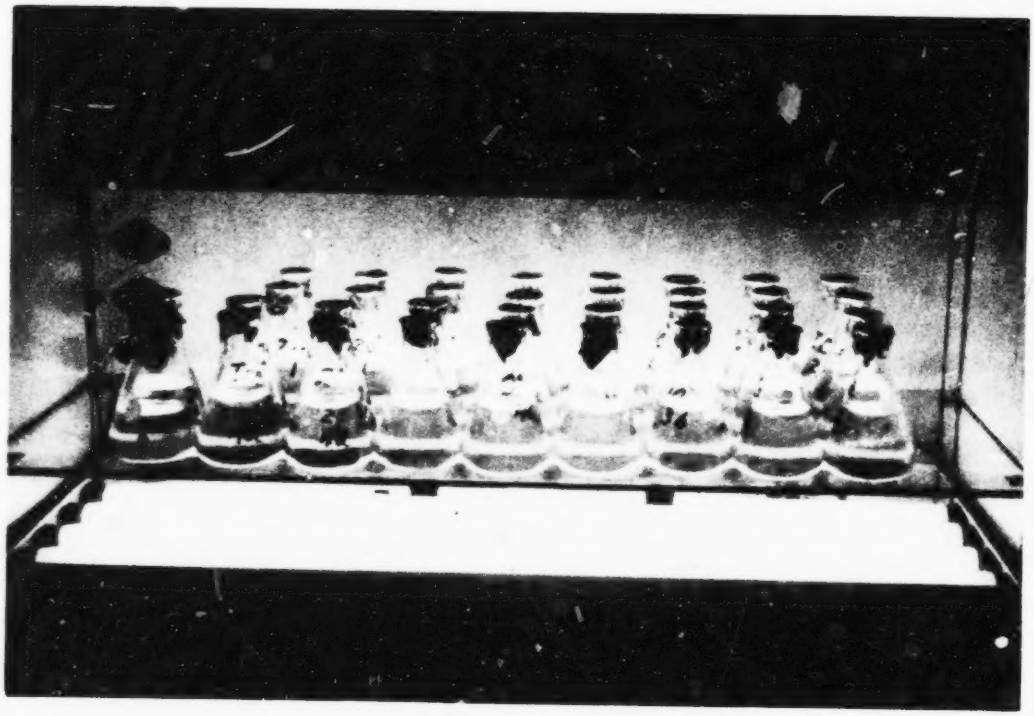

FIGURE I. VIEW OF INCUBATOR, ERLENMEYER FLASKS SHOWN ARE 250-ml SIZE

Culture vessels vary; 250 to 500-ml Erlenmeyer flasks are used in most cases although one-half gallon jugs are often used to hold oligotrophic waters where greater subsample volumes are required. Culture flasks are swirled every day when subsampled. When extraction procedures are required, subsamples are filtered through glass fiber paper and preserved with a slurry of $MgCO_3$. Filters not extracted immediately are frozen in darkness in desiccator jars containing silica gel. Direct reading eliminates these steps but is less sensitive and is employed only with denser algal crops found in eutrophic lakes or enriched river waters. Incubations are usually performed in duplicate although greater replication has been employed in assessments of oligotrophic waters.

The typical chlorophyll fluorescence response in such a batch test is often characterized by a sharp peak within the first week. Following the peak the chlorophyll fluorescence may decline sharply reflecting a shift from protein synthesis to that of carbohydrates as the culture ages under a nutrient limited condition

(Fogge 1965). The peak fluorescence achieved is termed the Algal Growth Potential (AGP) value. These peak chlorophyll levels do not necessarily occur in association with peak cell counts; diatoms have been observed to continue division becoming progressively smaller and paler while chlorophyll levels remained more nearly uniform. Chlorophyll peaks do appear to relate to major nutrient abundance – particularly nitrogen, an element essential to protein synthesis.

Chlorophyll concentrations are one way of quantifying standing phytoplankton crops. Other measures include the microscopic techniques such as cell counts, and dimensioning for cell volume, gravimetric techniques such as dry weight or volumetric measurements such as packed cell volume. Chlorophyll a, common to all plants, can also be used to predict primary production levels using the assimilation ratio concept (Ryther and Yentsch 1957, Bain 1968). Furthermore, chlorophyll is a direct measure of the green color, red tides, or surface scum or benthic growths which people find aesthetically objectionable in many eutrophic waters.

Standing crop levels may be more meaningful in describing the state of eutrophy or response to nutrient additions or dilutions in natural waters. Growth rates are not strongly suggestive of nutrition above threshold values; this point requires no further documentation here but recent works by Thomas which deal with this may be unfamiliar to some (1967, 1968). Standing crop measurements expressed as chlorophyll appear to be reasonably reflective of carbon, nitrogen, and phosphorus content; several papers dealing with chlorophyll as a measure of biomass have appeared recently which bear out earlier work on this (Weber and McFarland 1969, Keup and Stewart 1966). Such relationships may be as useful in predicting algal crops in eutrophic environments as assimilation ratios are in predicting primary productivity.

Examples of algal assays from various natural waters are shown in Figures 2 and 3. Figure 2 illustrates differences in algal response found in the three major tributaries of the San Joaquin River. The higher response (i.e., chlorophyll peak) of the Tuolumne and Stanislaus rivers reflects the greater nutrient content of these sewage and agricultural waste enriched streams in contrast to the cleaner Merced River. A similar illustration is provided by Figure 3 which depicts algal response in samples taken from various points along the Tuolumne River.

Figure 4 arrays algal chlorophyll a peaks (AGP values based on fluorescence) and total nitrogen concentration in San Joaquin Valley streams gathered from field data and replicated laboratory incubations during July, August, September, and October 1967 and June and September 1968.* Total nitrogen values are plotted because it is assumed that all or most inorganic forms found in field sampling are converted to algae in the assay tests and that all or most organic forms are comprised of algal cells from the field sample. Areas sampled range from the tidal San Joaquin near Stockton to the fresh clean flowing waters of the river near Friant Dam and include stations at the mouth of the three major tributaries and a major agricultural drain, Salt Slough. Algal growth response appears to reflect the increasing nitrogen content of these rivers as they flow toward the San Joaquin Delta.

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*Chlorophyll fluorescence (F) was converted to chlorophyll a values (α) expressed in micrograms per liter by the formula $\alpha = 0.55F$. This formula was developed from fluorometer and spectrophotometer readings.

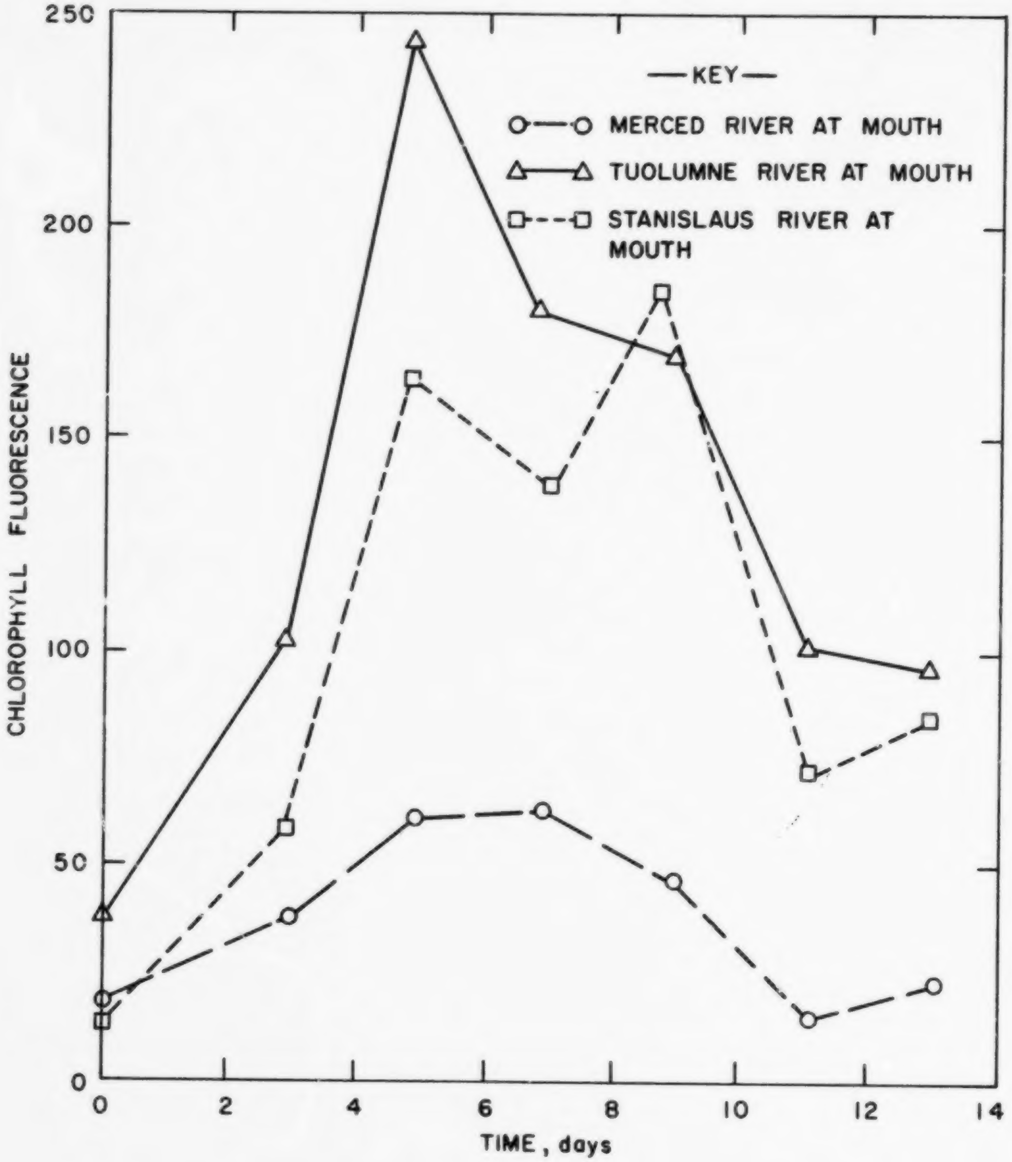

FIGURE 2. ALGAL GROWTH POTENTIAL, SAN JOAQUIN RIVER TRIBUTARIES, OCTOBER 1967

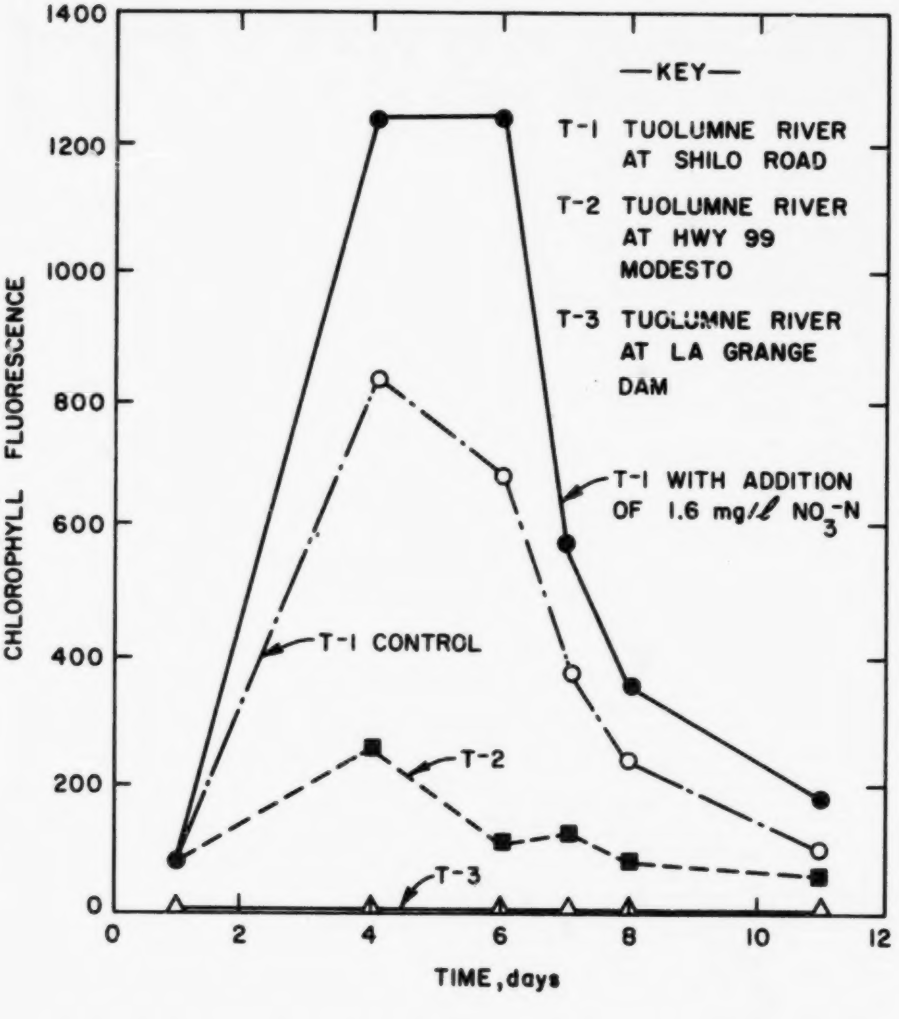

FIGURE 3. ALGAL GROWTH POTENTIAL, TUOLUMNE RIVER SURVEY, OCTOBER 1968

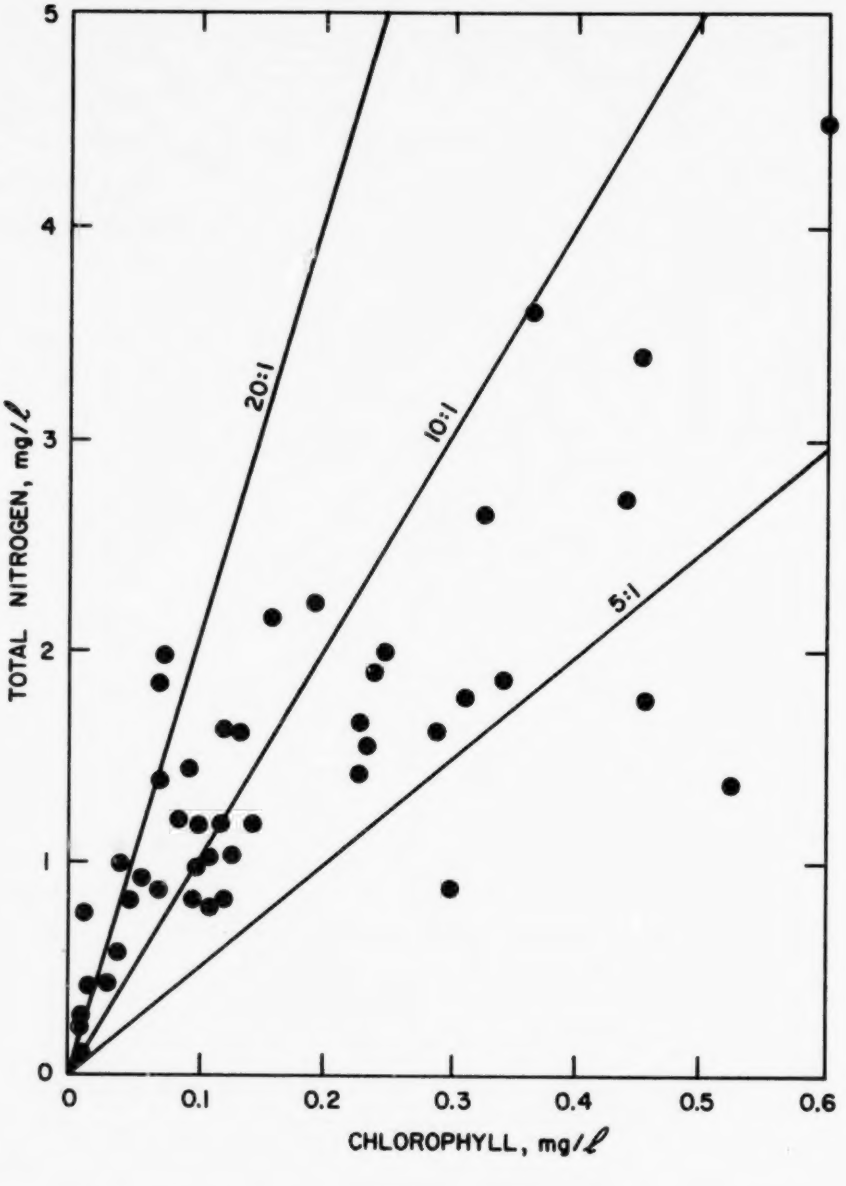

FIGURE 4. ALGAL GROWTH POTENTIAL AND TOTAL NITROGEN

Algal crops generally contain 50% carbon and 5-10% nitrogen on a dry weight basis and lesser amounts (1-2%) of phosphorus (McAllister et al. 1964; Parsons et al. 1961). Comparisons of chlorophyll a (α) values with organic nitrogen (N) from algal cultures indicate nitrogen/chlorophyll a ratios of 5-10:1 are approximated in healthy cells on a weight basis (Parsons et al. 1961; Manny 1969). On this basis a pure healthy algal culture should generate such a N/α ratio at the chlorophyll a peak if nitrogen is a limiting nutrient. The San Joaquin Valley stream data shown in Figure 4 generally fall between N/α ratios of 5:1 – 20:1; half of the data points fall between the ratios indicated for healthy algae. Only 3 points fall below the 5:1 line; these data could result from subsampling difficulties caused by clumping of algal cells. Ratios of nitrogen/chlorophyll greater than 10 suggest the presence of nonchlorophyll-bearing organic matter (zooplankton, bacteria, detritus, or soluble organics) or a failure of the culture to utilize all of the nitrogen.

A statistical analysis of the AGP data shown in Figure 4 yields the linear regression $N = 0.0033\,\alpha + 0.82$ where N is total nitrogen in mg/ℓ and α is chlorophyll a in μg/ℓ. Correlation was highly significant ($R = 0.7$), some data scatter is expected considering there is analytical and sampling error in both N and α. The mean total nitrogen for the 44 data points was 1.51 ± 0.18 mg/ℓ. Confidence limits (95%) of the mean N and slope (± 0.0009) yield N/α range of 4.6 – 10.8, approximately the same as the range for algal cells reported in literature.

Other examples of use of the procedure for nutrient response evaluation are illustrated in Figures 5 through 8. Figure 5 is an example from in situ incubations of San Joaquin Delta waters, including controls and samples which received nitrate-nitrogen additions (Bain et al. 1968). Figure 6 depicts both nitrate-nitrogen and chlorophyll fluorescence changes observed in an in situ incubation of San Francisco Bay water (Bain et al. 1968). Figure 7 depicts results from laboratory incubations of American River water with native water controls and samples inoculated with either nitrate-nitrogen ($NaNO_3$) and phosphate (K_2PO_4); Figure 8 illustrates results from a similar experiment using sewage as an inoculum in American River water (FWPCA). Other such experiments have been performed with agricultural and industrial wastes.

The procedure has been employed with success in assessing the relative effect of dilution of eutrophic lake waters with river water of lesser measured nutrient content (FWPCA 1968). Such an assay result is depicted in Figure 9. Confirming results were achieved through ^{14}C experiments conducted by Goldman (1968). Other algal fluorescent experiments in progress are designed to assess relative effect of denitrification treatment of San Joaquin Valley agricultural tile drainage on biostimulation when mixed with San Francisco Bay waters.

In summary, a simple batch type assay procedure has been employed for three years which appears to be reasonably versatile and capable of replication in situ or under laboratory conditions. Crops of native algal flora are measured in terms of chlorophyll fluorescence over a two-week test. Peak crops achieved in the test may be compared to nutrient levels or with results from other waters. Comparative testing of many waters has given a perspective to the results obtained with this assay procedure. Some interpretive applications are possible for use in predicting effects of waste disposal or flow regulation practices.

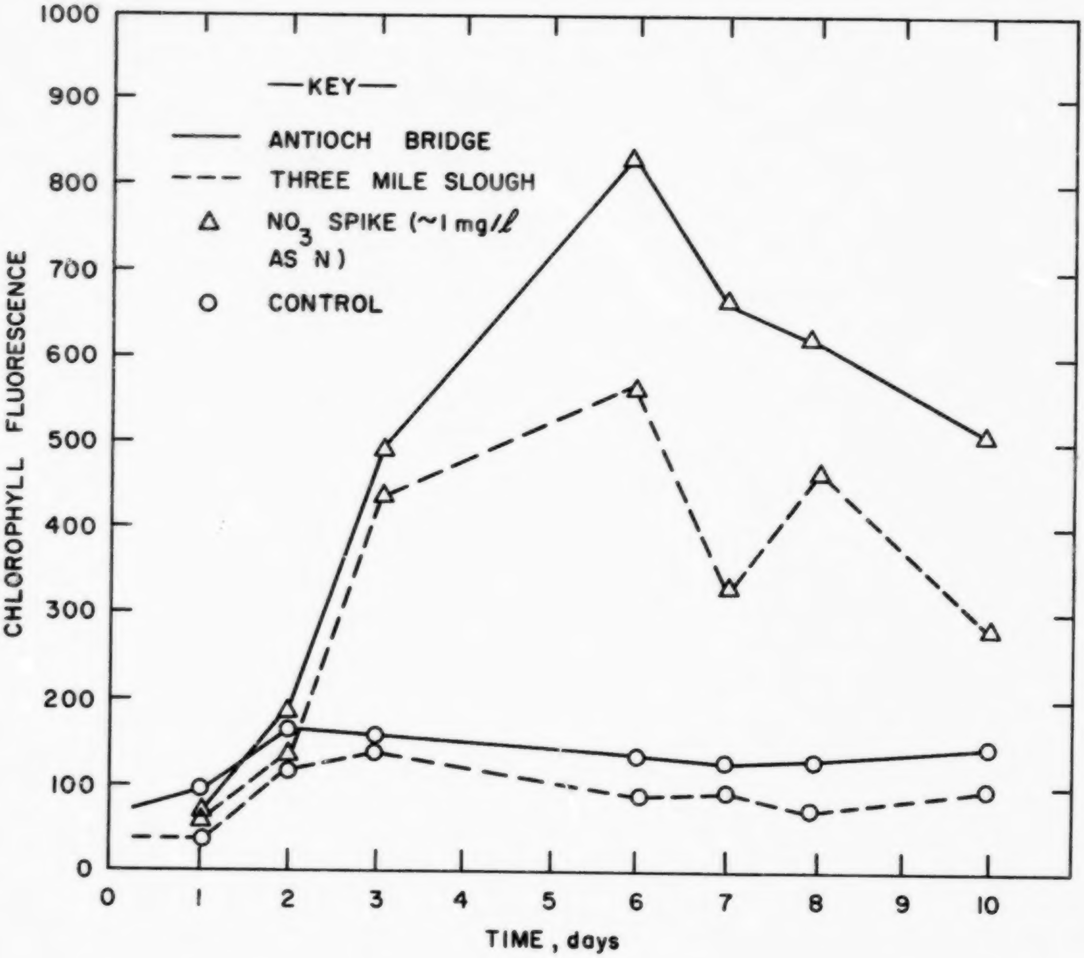

FIGURE 5. CHAMBER STUDIES, WESTERN DELTA

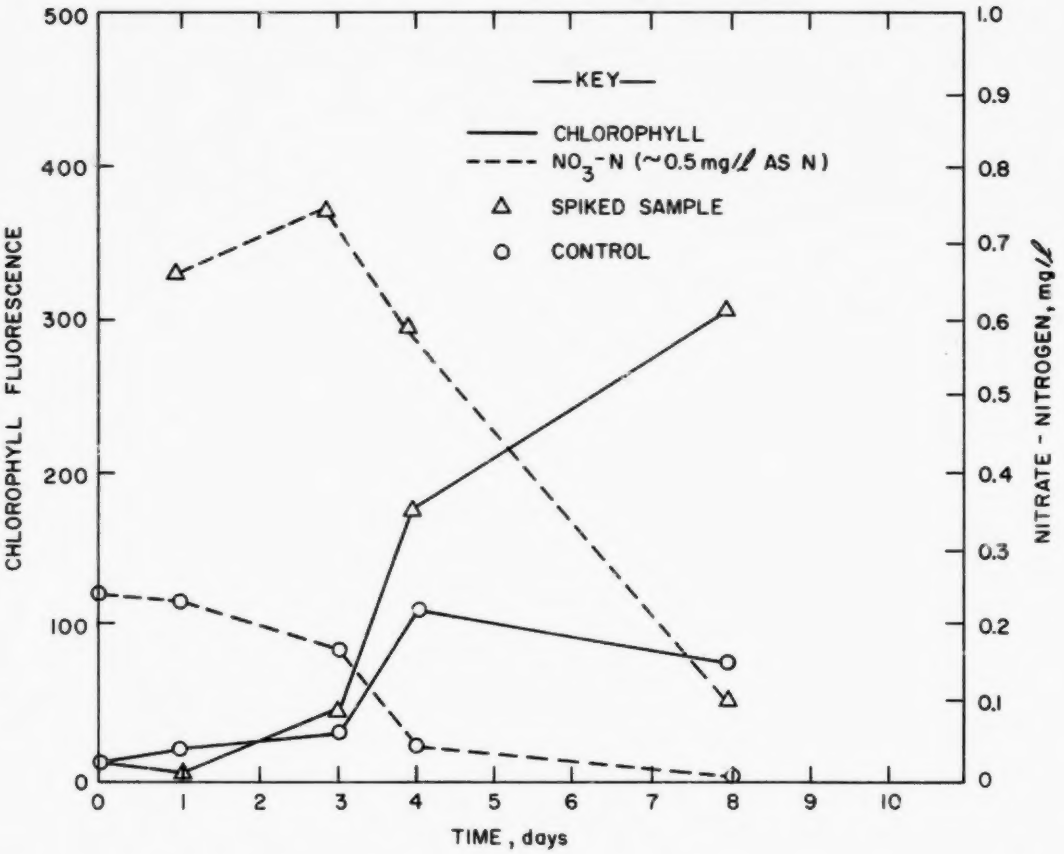

FIGURE 6. CHAMBER STUDIES, CARQUINEZ STRAITS

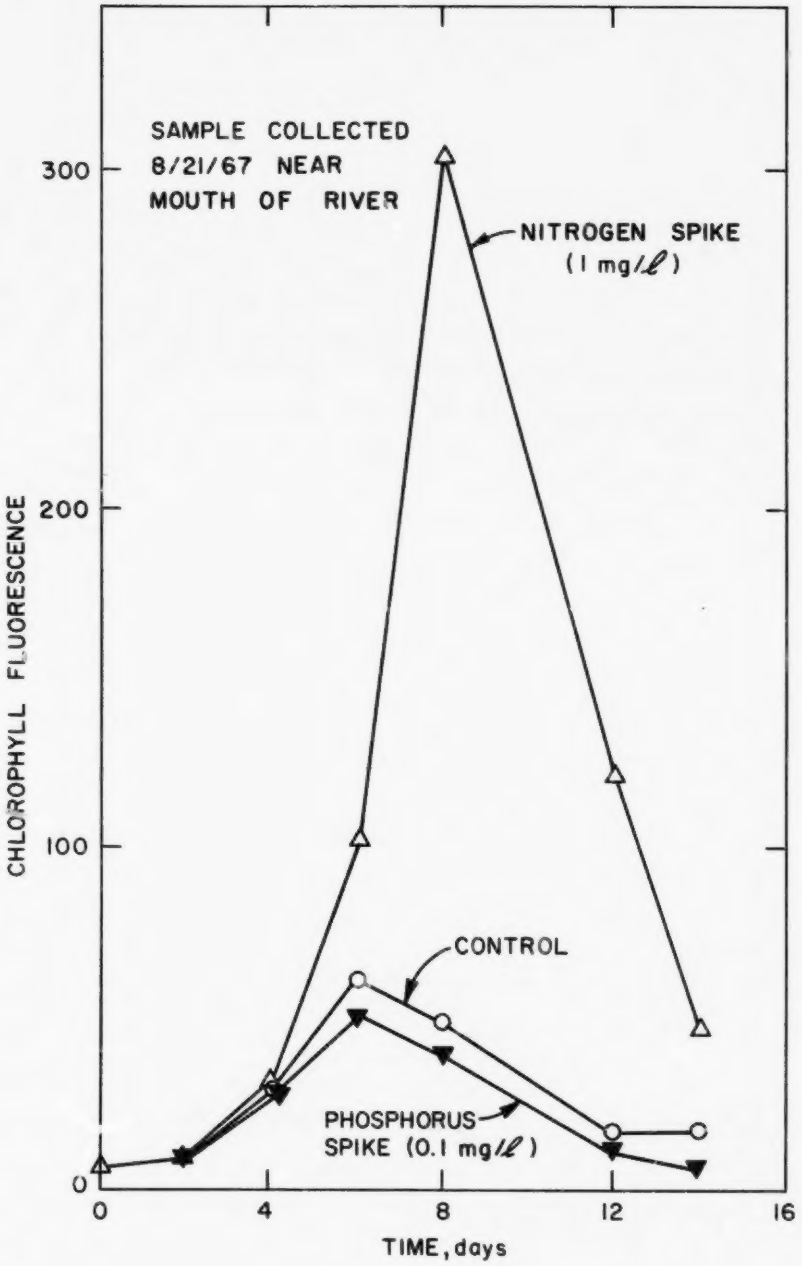

FIGURE 7. ALGAL GROWTH POTENTIAL, LOWER AMERICAN RIVER

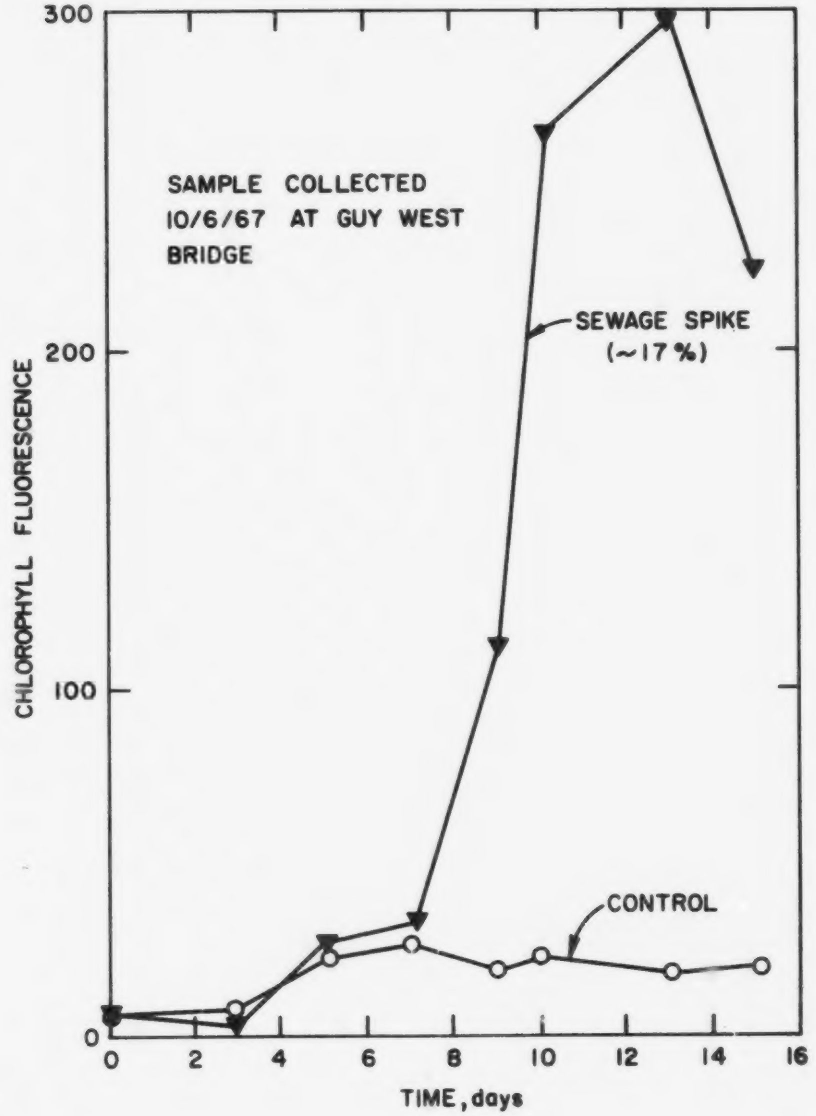

FIGURE 8. ALGAL GROWTH POTENTIAL, LOWER AMERICAN RIVER

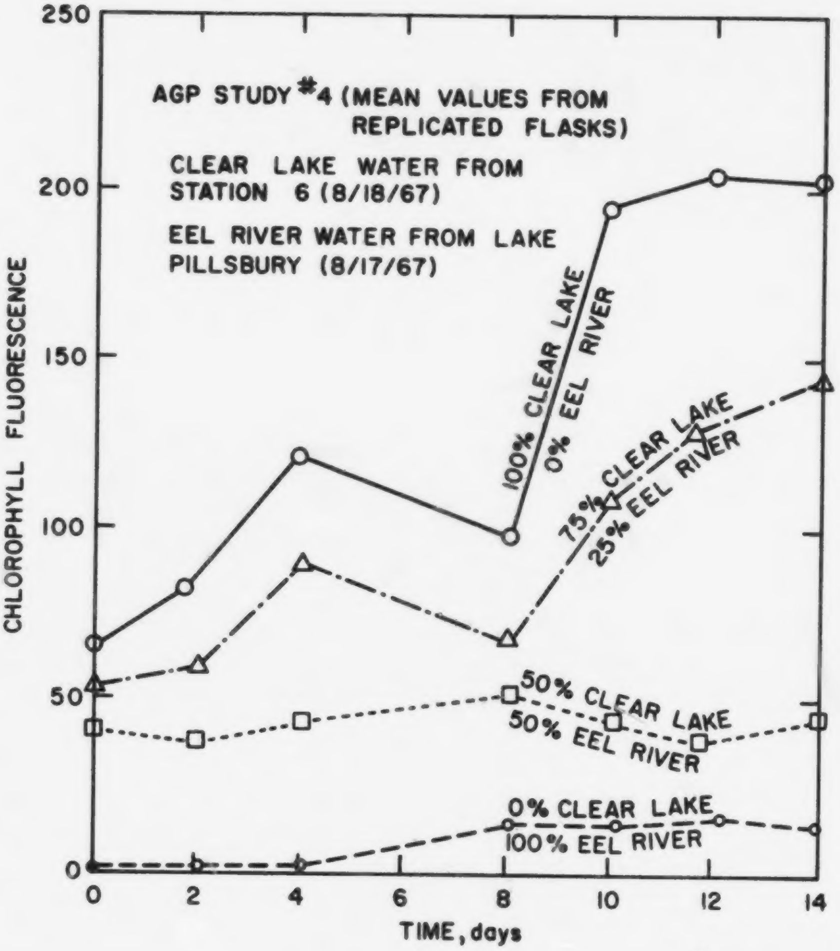

FIGURE 9. ALGAL GROWTH POTENTIAL, CLEAR LAKE & EEL RIVER

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APPENDIX – CHLOROPHYLL FLUORESCENCE EXTRACTION PROCEDURE

Principle

The water sample is filtered through a glass fiber filter and preserved by magnesium carbonate. The filter is kept frozen in a dark refrigerator. Extraction of the chlorophyll is accomplished with acetone by grinding in a tissue grinder, centrifuging, and reading the solution using a fluorometer or a spectrophotometer. Chlorophyll a is distinguished from phaeophytin by a second reading after addition of HCl.

Apparatus

Tissue Grinder, Powered by an electric motor.

Fluorometer; G. K. Turner with blue lamp #110-853, primary filter CS-5-60, and secondary filter CS-2-60.

Spectrophotometer; capable of using 1, 5, or 10 cm cuvettes and measuring wavelengths at 630, 645, and 665 mμ.

Small individual desiccators.

Glass filter papers, Whatman GF-C, 4.25 cm.

Millipore filter unit capable of using 4.25 cm filter paper.

Vacuum system capable of control to 5 in. mercury.

Reagents

Acetone Solution, 90%:

Pour into a 1-liter mixing cylinder 900 mℓ reagent grade acetone. Dilute to 1000 mℓ using deionized water.

Hydrochloric Acid, 1N

Using a mixing cylinder, dilute 8 mℓ A. R. grade concentrated HCl to 100 mℓ.

Magnesium Carbonate, Saturated Solution

50 g A. R. grade $MgCO_3$ in deionized water diluted to 100 mℓ.

Filtration Procedure

Vacuum System:

The vacuum system must be adjusted such that the pressure is no more than 5 in. Hg when water is being filtered. Higher vacuums cause chlorophyll loss through the filter.

1. Set up the millipore filtration flask and filter holder with one circle of the glass filter paper on the unit.
2. Shake the water sample vigorously until all algal clumps have been broken up.
3. While the water solution is still in motion, pour an aliquot into a graduated cylinder. The aliquot size is based on the amount of chlorophyll in the sample.
4. Pour the water into the filter holder and immediately squirt in two eye droppers of magnesium carbonate saturated solution.
5. Rinse the cylinder with a few mℓ of deionized water and pour into filter holder.
6. Rinse down the walls of the filter holder and allow all of the water to go through the filter.
7. Remove the top portion of the filter holder.
8. Fold the filter paper in half so the algae is inside the fold.
9. Place the filter paper in one of the individual desiccator jars and place in freezer.

Extraction Procedure

1. Remove filter from desiccator jar and place in the mortar section of the tissue grinder.
2. Squirt in 4-5 mℓ 90% acetone solution.
3. Insert the Teflon pestle into the mortar and macerate the filter paper.

   CAUTION: Do not let the solution get too hot as the heat can destroy the chlorophyll.

4. Rinse off the pestle into the mortar using a few mℓ of 90% acetone solution.
5. Dilute to volume in the mortar using the 90% acetone solution.

   or

   Transfer the solution quantitatively to a 15-mℓ centrifuge tube and dilute to 15 mℓ with 90% acetone.

6. Shake the sample for 1 minute and allow to set in a dark place for a minimum time of 20 minutes.

7. Shake the solution well again after the 20 minutes is up and centrifuge until clear (about 5 minutes).

8. Transfer the supernatant solution to a quartz tube and read the fluorescence in a Turner Fluorometer using the 3X, 10X, or 30X scale. If the emission is off-scale, then dilute the sample with 90% acetone.

   or

   Measure the solution in a spectrophotometer at 630, 645, and 665 mμ.

9. Add two drops of lN HCl to cuvette; mix, and reread the fluorescence.

   or

   If using the spectrophotometer, add two drops of lN HCl to the cuvette; mix, and reread the absorbance at 665 mμ only.

10. Reagent blanks should periodically be run through the procedure to ensure no fluorescing compounds are entering the system other than the chlorophyll.

KINETIC ASSESSMENT OF ALGAL GROWTH

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INTRODUCTION

Increasing attention worldwide is being focused upon the excess enrichment or eutrophication of natural bodies of water such as lakes, reservoirs, sluggish streams, and estuaries. The word eutrophication has become a scientific status symbol among conservationists and some limnologists; however, limited effort has been devoted to providing an adequate definition of the term. Nonetheless, a quantitative description of the term must be provided if solutions are ever to be developed for the presumed problems.

It is well known that nutrients such as nitrogen and phosphorus are required for algal metabolism. However, the question remains as to whether nitrogen or phosphorus usually or frequently limit the algal growth rate or standing stock in most receiving waters. To effectively control the rate of eutrophication of our natural waters this question must be answered. It is not enough to remove nitrogen and phosphorus from wastewaters in the hope that this alone will significantly reduce the concentration of nitrogen or phosphorus in the receiving waters or the growth rate or standing stock of plankton in the waters of concern.

There is an urgent need to develop methods or techniques for assessing the biostimulatory character of waste effluents, natural runoff, etc., so that one can estimate the level of algal populations one might expect for a given level of rate-limiting substance in the receiving water. In no instance is this need more urgent than in the case of Lake Tahoe, which as one of the clearest alpine lakes in the world is a unique national asset. Although action to remove sewage effluents from the basin is well under way, urban activity in the area is rapidly increasing and it is not known whether other waste material or drainage to the lake should be controlled to limit the rate of eutrophication.

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THE PROBLEM

To develop a realistic waste management program for a body of water assumed to be excessively enriched by nutrients or other biostimulatory materials, one must be able to develop data that will permit the accomplishment of the following objectives:

1. To estimate as accurately as possible the background or near steady-state level of objectionable algae that will be supported by a specific concentration of rate-limiting substance.

2. To estimate the concentration of the rate-limiting substance that will produce an algal bloom, which can be defined as a pulse or increase in growth rate, μ, of some specific value – say 2.0 day^{-1} (i.e., 200 percent increase in cell mass per day).

It should be noted that the foregoing definition considers the test substances to be evaluated as the rate limiting nutrient (all other nutrient and energy sources in excess). Therefore, estimates obtained in the laboratory represent the maximum concentration of the test organism (particular alga) that can be maintained in a given system simulating the prototype and the maximum rate of growth that can be attributed to the concentration of the specific nutrient in the receiving water.

LABORATORY TEST – MODEL PROTOTYPE SIMULATION

Ideally, results obtained in the laboratory should apply as accurately as possible to the specific practical problem and situation of concern. This is, or should be, the object of every bioassay procedure intended to provide a basis for establishing allowable concentrations of contaminants in the environment. Probably the best example of this approach to "real" problems is the classic Biochemical Oxygen Demand (BOD) test and its use in the engineering design of processes, and in the kinetic modeling (somewhat oversimplified) of oxygen concentrations (oxygen sag curve) in a stream as a function of time, organic loading (BOD), and numerous other physical and biological factors. The lack of precise simulation of the prototype by the laboratory assay method is well understood; nonetheless the laboratory assay method attempts to model the prototype and does provide a crude approximation of the real situation which is very valuable in the conceptual analysis of the problem and in sorting out practical design solutions. It appears to the writers that this is what is needed for the quantitative assessment of the biostimulation or enrichment potential of waste discharges including both energy (carbon) and nutrient bearing wastes.

ASSAY METHOD – THEORY AND RATIONALE

Two types of assay methods, the batch and continuous flow culture assay, must both be considered as to their efficacy in simulating the prototype, the accuracy of the method, and the interpretability of the data obtained.

Batch Culture – Plug Flow Model

The traditional microbiological batch culture, transient growth-rate assay procedure, is in essence a laboratory model of a continuous-plug-flow reactor. The nearest thing to a plug-flow reactor in the real world is a long, lined river or canal which can be considered to have limited longitudinal mixing and no sources or sinks of constituents of concern below the point of major waste discharge. For an ideal plug-flow system, the fluid is considered to have zero longitudinal mixing. This is illustrated schematically in Figure 1. The residence time of all molecules in a plug-flow system is identical; simply the reactor volume, V, divided by the throughput flow, F (i.e., V/F). The concentration of the rate-limiting nutrient changes with distance (or time) in the reactor and this concentration profile is shown also in Figure 1.

The batch growth culture method follows the concentration of cells in an inoculated flask starting with an initial substrate with all energy sources, nutrients, growth factors, etc. in excess, except the one substance of concern which is considered to be the growth-limiting substance.

Figure 2 shows a graph of the typical microbiological growth curve with the concentration-time curve for the growth-rate-limiting nutrient (S) also shown.

There are a variety of ways to describe the characteristics of the batch culture growth curve. One characteristic of the curve that has been suggested has been the maximum cell concentration, $\hat{X}$, reached by a particular organism and for a specific time, substrate, and initial concentration, S_o.

If the concern about nutrient concentrations present in the environment is their effect on growth rate of specific algae, then a better parameter of the curve might be the maximum growth rate obtained with the test substrate. This characteristic is shown in Figure 2 as $\hat{\mu}_b$ (maximum growth rate – batch). However, it has been established by Michaelis-Menten (1913), Monod (1949), Caperon (1965), Maddux (1963), Williams (1965), Jannasch (1966), Dugdale (1967), Pearson (1966), and others that the specific growth rate of microorganisms (including algae) is either a first order or first order–zero order (Michaelis-Menten) function of the rate-limiting nutrient concentration. That is

$$\mu = k\,S \qquad \text{or} \qquad \mu = \frac{\hat{\mu}\,S}{K_s + S}$$

where

k = first order – rate constant, time^{-1}

μ = specific growth rate $\frac{\text{grams cells produced}}{\text{gram cells-day}}$, i.e., time^{-1}

$\hat{\mu}$ = maximum specific growth rate

K_s = substrate concentration at one-half the maximum specific growth rate

S = rate-limiting substrate concentration.

Examination of Figure 2 indicates that one can estimate the maximum growth rate for a batch culture; however, this maximum growth rate occurs at an undetermined but lesser concentration of the rate-limiting nutrient than was in the original

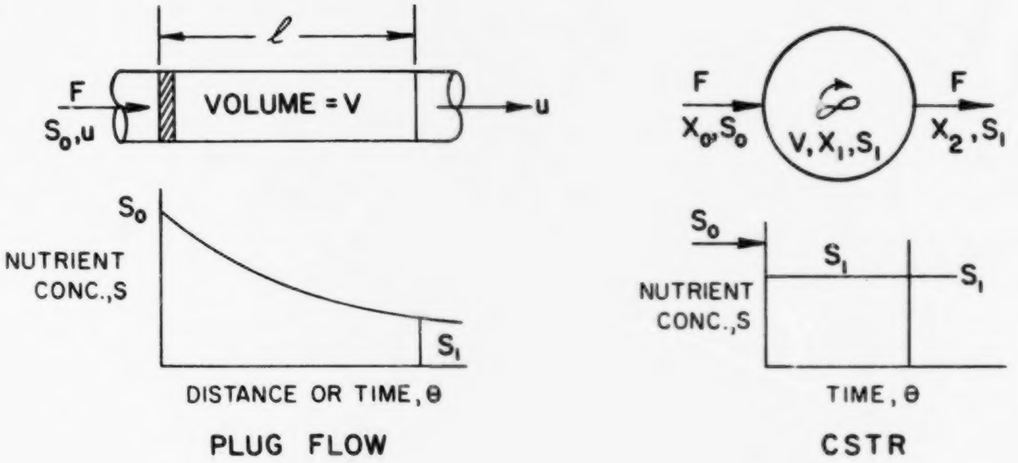

ℓ
F
VOLUME = V
S_0,u
u
S_0
NUTRIENT CONC., S
S_1
DISTANCE OR TIME, θ
PLUG FLOW
F
X_0, S_0
V, X_1, S_1
F
X_2, S_1
S_0
S_1
S_1
NUTRIENT CONC., S
TIME, θ
CSTR

FIGURE I. REACTION SYSTEMS (MODELS)

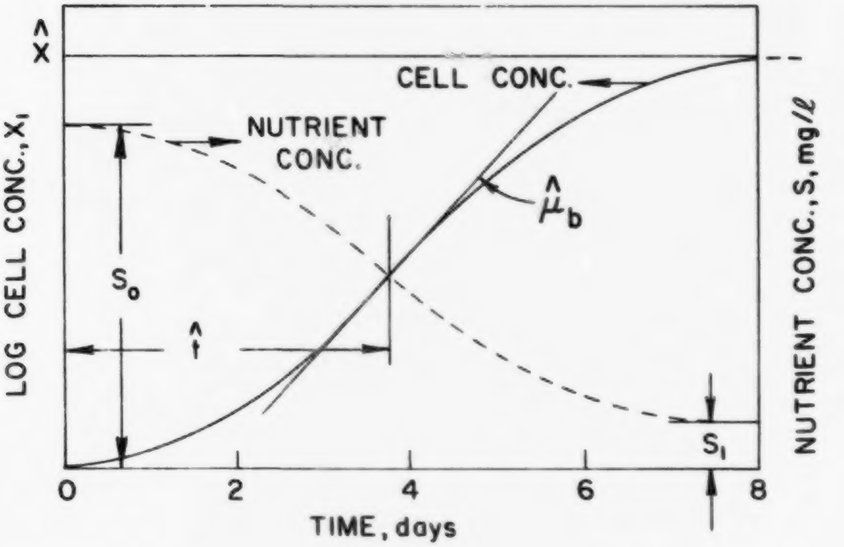


FIGURE 2. TYPICAL MICROBIOLOGICAL GROWTH CURVE, BATCH

sample, S_o. This maximum growth rate should indicate the maximum rate that the algal culture can utilize the nutrient present in the sample; however, it does not define the precise relationship between growth rate and substrate concentration — and an estimate of this is needed.

Possibly, the maximum growth rate batch and the time to reach the maximum growth rate can be correlated to the substrate concentration; however, this has yet to be done, and for a transient system such as the batch culture, it is not a simple task.

Continuous Flow Culture — (Chemostats) — CSTR Model

During the past fifteen or so years the culturing of biological populations on a continuous flow, steady-state basis has become popular. Moreover, it has been for good reason because in a continuous flow, steady-state system, the biological population can be kept at steady state conditions (or nearly so) over a wide range of growth rates. One can study the behavior and characteristics of an organism growing as slowly as 0.01 day^{-1} (200 percent growth of new cell mass per day, depending upon the organism). It is also quite significant that one finds gross differences in the behavior, characteristics, and even products of organism growth depending upon an organism's growth rate.

One of the major advantages of a continuous flow—steady state assay method is that it permits one to determine, under laboratory conditions, the level of standing stock of any organism or mixtures of organisms that can develop in a given hydraulic reaction system (a particular hydraulic residence time, i.e., detention period) for any concentration of the rate-limiting nutrient. This level then, is the maximum concentration of a particular organism that can be supported by the given concentration of nutrient. Moreover, one can determine from the test procedure what concentration of the rate-limiting nutrient is required to produce any given increase in the growth rate (2 times, 5 times, etc.) at any specific level of cells (i.e., standing stock). The basis for the foregoing comments is presented in the following summary of the kinetics of biological systems.

Kinetics

In accordance with the previously described basis for relating growth of microorganisms to the concentration of rate-limiting nutrient, it has been shown that the specific growth rate, μ, is best described by the Michaelis-Menten or Monod model as follows:

$$\mu = \frac{\hat{\mu}\, S_1}{K_s + S_1} \tag{1}$$

where the terms are as defined previously. The specific growth rate is first order with respect to the rate-limiting nutrient concentration, S_1, at low levels of nutrient concentration; that is:

$$\mu = kS_1, \qquad \text{when} \qquad S_1 \to 0 \;. \tag{2}$$

If one makes a nutrient and cellular materials balance on a simple chemostat reactor system (CSTR) one obtains the following expressions for nutrient and cellular growth, respectively.

$$q = \frac{F(S_o - S_1)}{X_1 V} = \frac{S_o - S_1}{X_1 \theta} \qquad (3)$$

where

q = specific nutrient removal velocity $\frac{\text{grams nutrient removed}}{\text{gram cells-day}} \left(\text{day}^{-1}\right)$

F = waste flow rate

S_o = concentration of rate-limiting nutrient in untreated feed

S_1 = concentration of rate-limiting nutrient in the reaction system

X_1 = concentration of cells in reaction system

V = volume of reaction system

θ = hydraulic residence time of system, i.e., $\theta = V/F$

and,

$$\frac{FX_2}{X_1 V} = \frac{1}{\theta_c} = \mu - k_d \qquad (4)$$

where the new terms are as follows:

X_2 = concentration of cells leaving the reaction system

θ_c = cellular residence time (i.e., mean cell age in system)

$\frac{1}{\theta_c}$ = net cellular growth rate

k_d = specific decay rate $\left(\text{i.e., } \frac{\text{grams cells destroyed (lysed, etc.)}}{\text{gram cells-day}}\right)$.

It can be shown that Equations 1 and 4 can be written

$$q = \frac{\hat{\mu}\, S_1}{Y(K_s + S_1)} \qquad (5)$$

$$\frac{FX_2}{X_1 V} = \frac{1}{\theta_c} = Yq - k_d \qquad (6)$$

where Y = yield coefficient $\left(\text{i.e., } \frac{\text{grams cells produced}}{\text{grams nutrient removed}}\right)$.

This can be deduced from a simple continuity or dimensional analysis as follows:

$$\mu = Y \times q \tag{7}$$

$$\frac{\text{grams cells produced}}{\text{gram cells-day}} = \frac{\text{grams cells produced}}{\cancel{\text{grams nutrient removed}}} \times \frac{\cancel{\text{grams nutrient removed}}}{\text{gram cells-day}} .$$

Thus, by conducting a series of steady state experiments for each organism (or group of organisms) at three concentrations of nutrient (S_1), one can determine the magnitude of the rate and system constants, μ, K_s, Y, and k_d from analysis of data collected for each of the measurable parameters, F, S_o, S_1, X_1, X_2, and 0. The data processing technique has been described in detail by Pearson (1966). Each of the three experiments should be at a different residence time, which will result in a different effluent concentration S_1 for a constant level of nutrient feed, S_o.

It should be readily apparent that if one determines the constants $\hat{\mu}$, and K_s in Equation 1, the specific growth rate for any level of nutrient concentration in the system can be predicted. That is,

$$\mu = \frac{\hat{\mu}\, S_1}{K_s + S_1} . \tag{1}$$

This information, coupled with the determined value of the constants Y and k_d, permits estimation of the net growth rate of the system, $1/\theta_c$.

Using Equation 3 and substituting μ/Y for q, one obtains

$$\mu = \frac{Y(S_o - S_1)}{X_1 \theta} \tag{8}$$

or transposing

$$X_1 = \frac{Y(S_o - S_1)}{\theta \mu} . \tag{9}$$

The relationship between the mean cell concentration X_1 and the residence time θ for a given nutrient concentration is obvious.

The kinetic description of biological systems and its application to eutrophication problems can be summarized as follows:

1. A given level of rate-limiting nutrient (i.e., $S_1 = NO_3^-$, $PO_4^{\equiv}$, etc.) in the receiving water determines the specific growth rate, μ, that can develop from that rate-limiting nutrient concentration, i.e., $\mu = f(S_1)$.

2. For a given level of S and μ, and yield constant, Y, the mean cell concentration, X_1, (standing stock) is determined by the residence time in the system. This is obvious from inspection of Equation 9.

3. One can estimate the net or gross cellular growth rate that will result from any concentration of rate-limiting nutrient concentration once the rate constants and coefficients have been determined for the organism and nutrient form (Equation 4).

It appears that this is the type of information needed for prediction of allowable levels of organisms (algae) and nutrients of concern in the receiving water. Moreover, these data are susceptible to laboratory evaluation. The accuracy of such predictions (simulation of the prototype) can only be assessed by determination in the laboratory of the kinetic characteristics described herein followed by assessment of the resulting conditions in the field.

EXPERIMENTAL METHODS

General

The algae used in both the batch and continuous-culture systems were Selanastrum gracile which were grown in a modified Bristols medium, harvested when growing in the log-phase, and washed twice to minimize nutrient carry-over. The cultures were incubated at 20°C and were exposed to about 170 ft-candles of light. The light source was 30-watt GE fluorescent lamps. The culture densities varied over a wide limit in the batch culture system and it is possible that light as well as the nutrient source may have been limiting. However, in the chemostat, the cell concentration was maintained at a level so that it is highly unlikely that light was limiting.

Cell mass determination was made by counting and calculating volume and mass from observations on cell size. All growth rate determinations are on a mass basis.

All samples tested were filtered through a 0.45-μ membrane filter to remove debris and extraneous algae. The diluent for all samples was Lake Tahoe water.

Batch Culture

Cell measurements were made about every other day and the maximum growth rate ($\hat{\mu}_b$) computed from the log phase growth data as well as the maximum cell concentration $\hat{X}$ from the plotted growth curve.

Figure 3 shows a photograph of the batch culture system employed.

FIGURE 3. BATCH CULTURE SYSTEM

Continuous Flow, CSTR

The chemostats were inoculated with the test alga at a concentration level of 20-50 cells/cu mm and fed continuously. Cell counts were made every two days and the experiment was continued for at least two residence times after an apparent steady state was reached (cell counts for 3 successive sampling periods (~ 6 days) within $\pm$ 20 percent and no trend indicated). To develop data for kinetic constant evaluation, at least three different residence times were made within the range $15 \le \theta \ge 5$ days.

Figure 4 shows a detailed sketch of the chemostat system and Figure 5 shows the reactor system in detail.

A detailed description of the experimental methods is reported in the First Progress Report, FWPCA No. WPD-48 (1968).

RESULTS

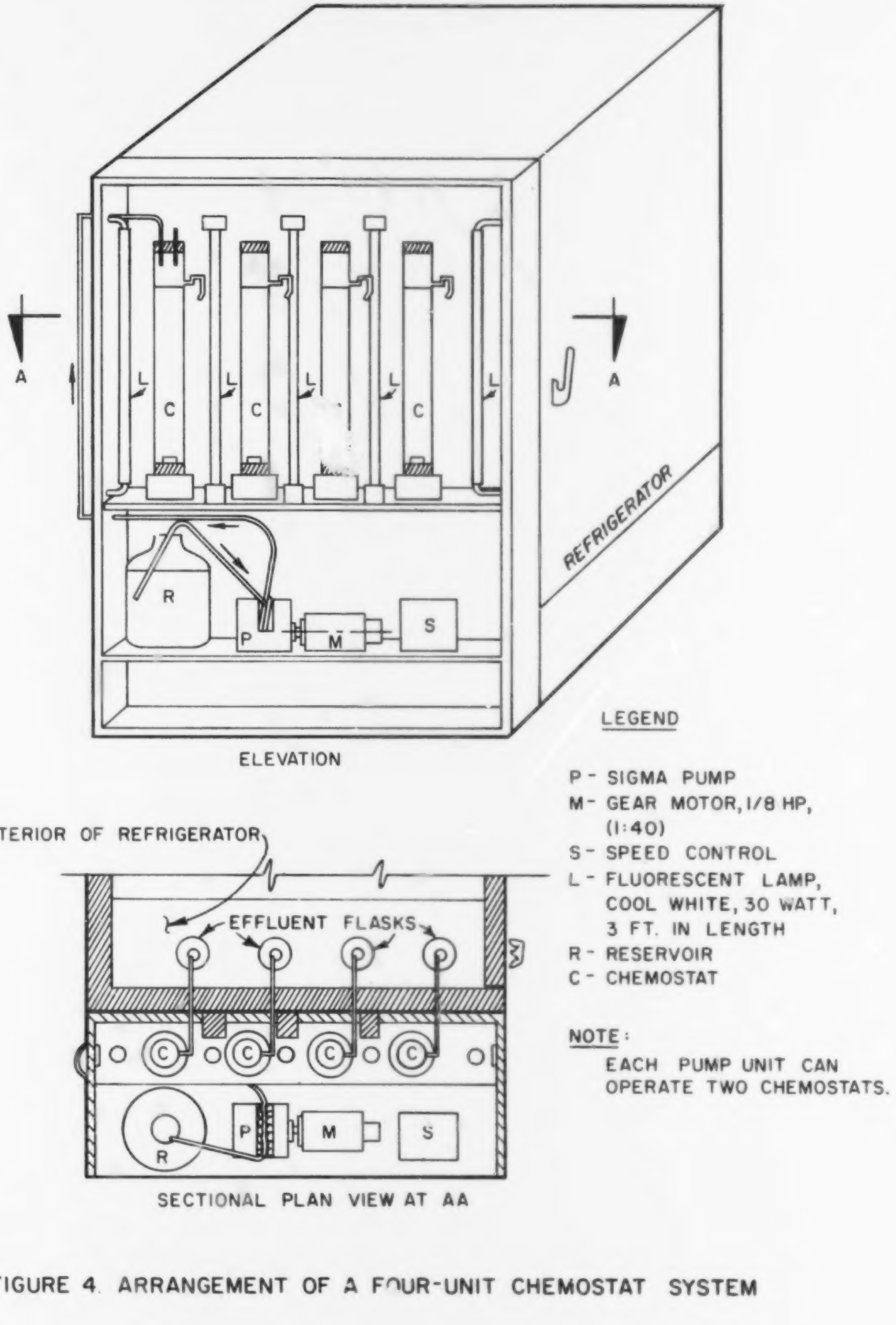
Batch Culture

Lake Tahoe Water: The diluent for all samples was Lake Tahoe water (LTW) which turned out to be a poor choice because of its substantial and apparently cyclical variation in biostimulatory properties. The seasonal variation in $\hat{\mu}_b$, the maximum batch growth rate, is shown in Figure 6. A significant difference (95 percent level of significance in growth response) was observed in LTW samples collected during late summer and early winter (June 6 — December 11), during the winter (December 12 — March 30), and during the spring (April). The mean obtained for the late summer and early winter LTW samples was 0.159 day^{-1}; and the mean $\hat{\mu}_b$ for the winter samples increased to 0.293 day^{-1}. When the spring LTW samples were assayed, the mean $\hat{\mu}_b$ had increased to 0.430 day^{-1}. This respresents a mean increase in growth response in LTW of almost 300 percent from late summer to the following spring.

Good precision was obtained in duplicate assays on LTW alone; therefore, it appears that little of the variation can be attributed to experimental error. Instead, the data strongly suggest that a significant change in growth response of LTW occurred with time.

Surface Runoff: Comparison of the growth rate response characteristics for four of the major creeks (Meeks, Ward, Incline, and Truckee-Trout) indicates either that the batch-type response test is relatively insensitive or that there is little difference in the response rate for different dilutions (1%, 10%, and 50%) or between individual creeks independent of dilution. The only notable exception is Truckee-Trout Creek, which because of its tributary area might be expected to have greater biostimulatory properties.

Table I presents a comparison — including a statistical assessment — of ranked means of maximum batch growth rate ($\hat{\mu}_b$) observed at three sample concentrations (1%, 10%, and 50%) for each creek. The underlined bracket for each creek includes the response rate values ($\hat{\mu}_b$) and sample concentrations which are not significantly different at a 95 percent confidence limit. Inspection of the table reveals that the only creek showing significantly different growth rates ($\hat{\mu}_b$) at all three waste concentrations was Truckee-Trout Creek. The growth rate of the 50 percent sample concentration for Incline Creek was the only other value significantly different from either the 1 percent or 10 percent sample concentrations.


A
A
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REFRIGERATOR
R
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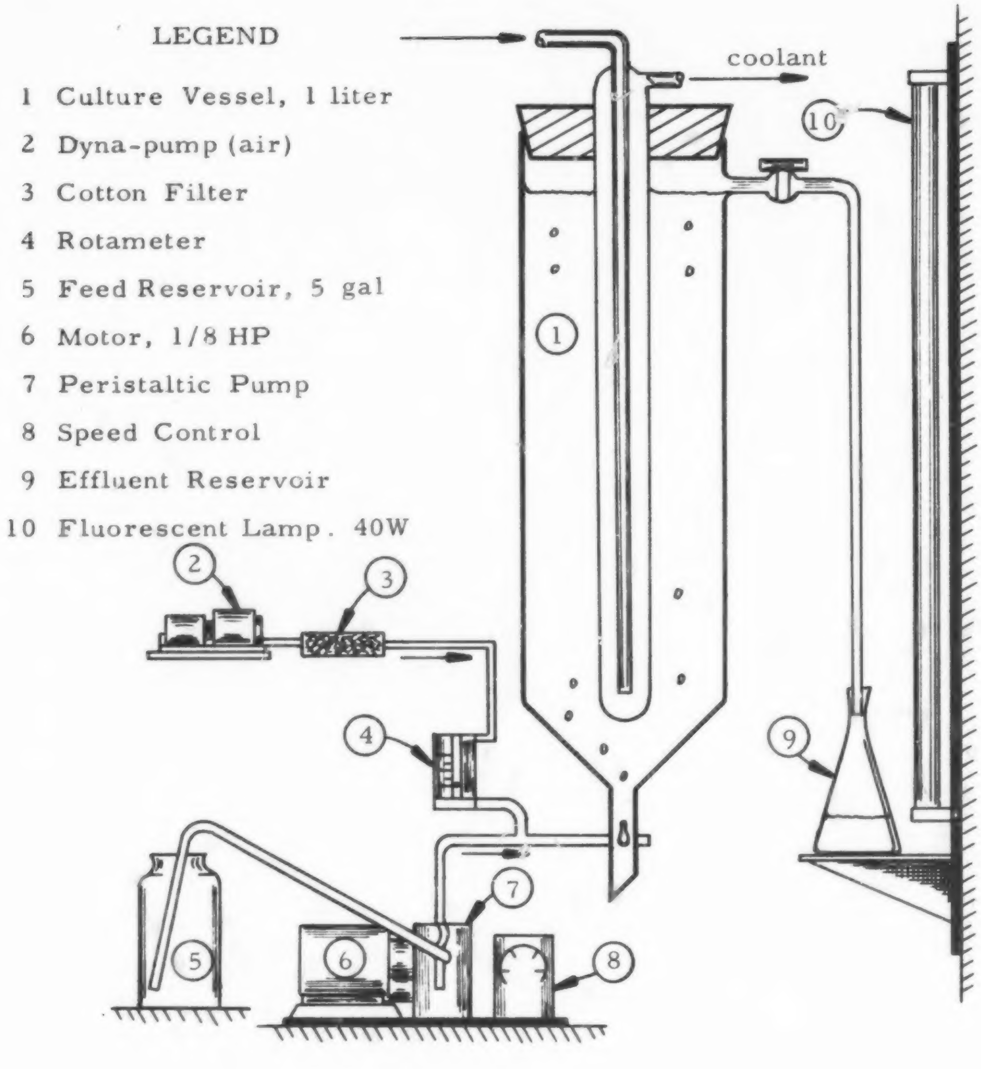
ELEVATION
INTERIOR OF REFRIGERATOR
EFFLUENT FLASKS
C
C
C
C
P
M
S
R
SECTIONAL PLAN VIEW AT AA

LEGEND

P - SIGMA PUMP
M - GEAR MOTOR, 1/8 HP, (1:40)
S - SPEED CONTROL
L - FLUORESCENT LAMP, COOL WHITE, 30 WATT, 3 FT. IN LENGTH
R - RESERVOIR
C - CHEMOSTAT

NOTE:
EACH PUMP UNIT CAN OPERATE TWO CHEMOSTATS.

FIGURE 4. ARRANGEMENT OF A FOUR-UNIT CHEMOSTAT SYSTEM



LEGEND

1 Culture Vessel, 1 liter
2 Dyna-pump (air)
3 Cotton Filter
4 Rotameter
5 Feed Reservoir, 5 gal
6 Motor, 1/8 HP
7 Peristaltic Pump
8 Speed Control
9 Effluent Reservoir
10 Fluorescent Lamp. 40W

FIGURE 5. FLOW DIAGRAM OF CHEMOSTAT SYSTEM

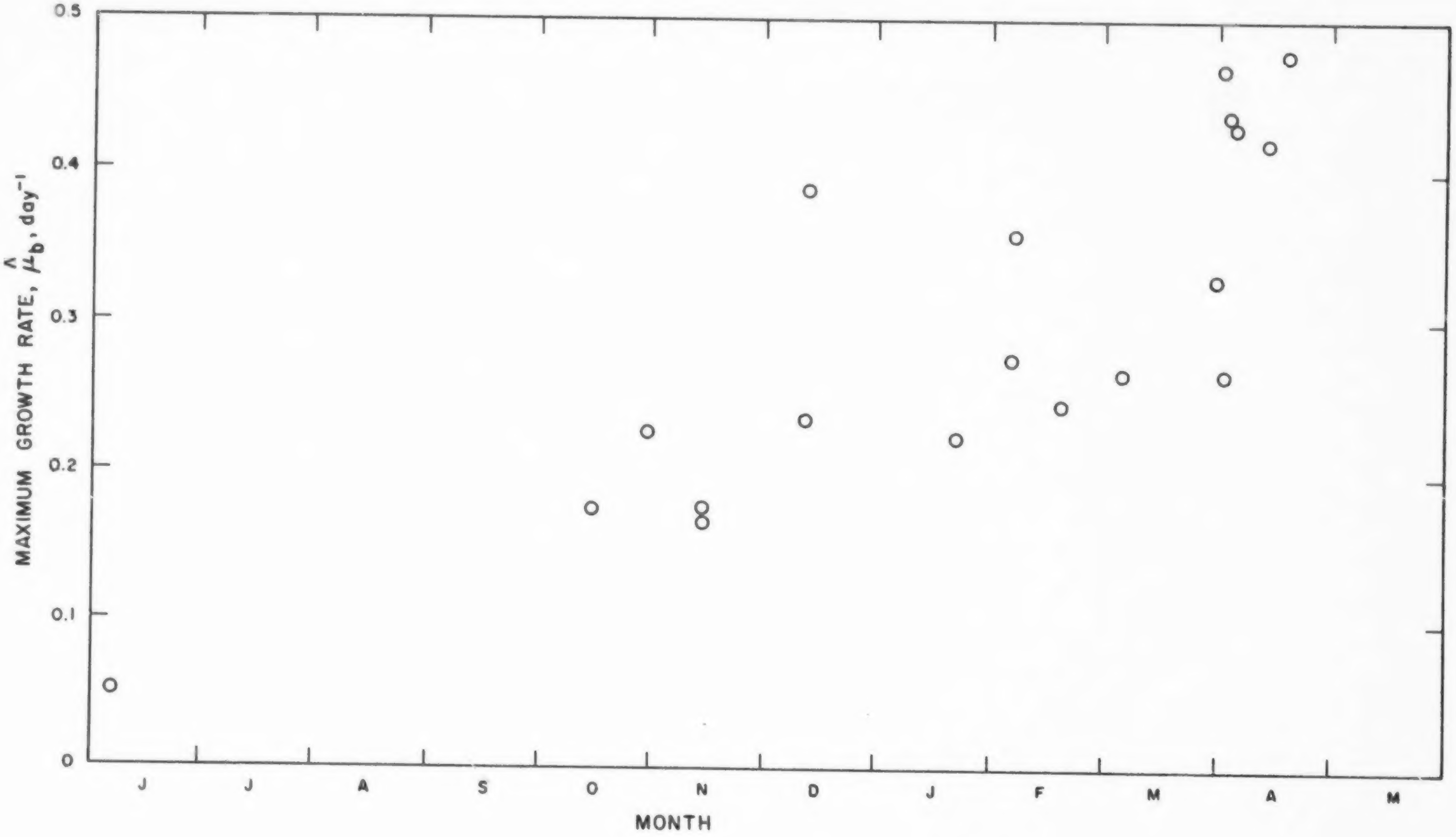


FIGURE 6. SEASONAL VARIATION IN THE MAXIMUM GROWTH RATE IN LTW ALONE

TABLE I

RANKED MEAN MAXIMUM GROWTH RATES, $\hat{\mu}_b$, CORRESPONDING SAMPLE CONCENTRATION AND STATISTICAL SIGNIFICANCE FOR "NATURAL" RUNOFF – BATCH ASSAYS

Creek	$\hat{\mu}_b$, day^{-1}; Sample Concentration, (%), and Statistical Significance; No Significant Difference at 95% Confidence Level		
Meeks	0.233 (10%)	0.243 (1%)	0.298 (50%)
Ward	0.175 (50%)	0.198 (10%)	0.238 (1%)
Incline	0.125 (1%)	0.208 (10%)	0.325 (50%)
Truckee-Trout	0.320 (1%)	0.610 (10%)	0.850 (50%)

NOTE: Samples not underscored are significantly different at 95% Confidence Level.

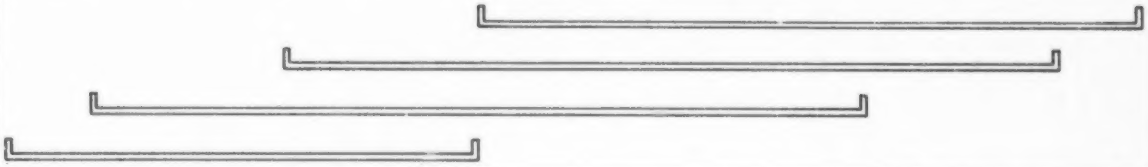
Waste Effluents: One of the principal objectives of this study was to assess the relative biostimulation level of a wide variety of nutrient sources. It was, therefore, not possible to study a single source in adequate detail to determine the variability in response on a statistically valid basis. Although a number of treatment plant effluents – Incline Village Extended Aeration and Reno's Activated Sludge plants – were assayed with sufficient replication to warrant statistical analyses of the observed data.

A comparison of the mean maximum growth rates obtained with sewage treatment plant effluents is shown in Table II, in which maximum growth rates that do not differ significantly at a 95 percent confidence level are underlined with a continuous line. Values not connected with a line differ significantly. Inspection of the table reveals that there was no statistically significant difference in the growth response rates between the Incline and Reno effluents except at the 50 percent concentration level. At this concentration the mean $\hat{\mu}_b$ for Reno activated sludge effluent was 0.94 day^{-1} compared to 0.56 day^{-1} for Incline effluent which was significantly different at a confidence level of 95 percent.

The growth response obtained in the assays on samples containing concentrations of inorganic nutrients (N and P) equivalent to that present in each effluent sample was generally much lower than that observed for the particular effluent and sample concentration. The only exception to this generalization occurred with the Reno activated sludge effluent samples. In this case the growth-rate response for equivalent chemical nutrients was generally equal to or greater than the growth rate obtained with the Reno effluent samples.

TABLE II

EFFLUENT MEAN MAXIMUM GROWTH RATES, $\hat{\mu}_b$, WITH REFERENCE TO SAMPLE CONCENTRATION AND EQUIVALENT N AND P CONCENTRATION IN LTW AND STATISTICAL SIGNIFICANCE

Source of Sample[a]	II	II	I	R	I	RI	RI	RI	II	R	I	R
Percent Sample	1	10	50	1	1	1	50	10	50	10	10	50
Mean $\hat{\mu}_b$	0.37	0.53	0.56	0.61	0.62	0.70	0.76	0.79	0.80	0.88	0.89	0.95
Number of Replications	7	7	5	6	5	7	7	7	7	6	5	6
Samples connected with Common Underline are not Significantly Different at P = 95%												

[a] Code

R = Reno Activated Sludge Plant

I = Incline Extended Aeration Plant

RI = Inorganic Nutrients (N and P) Equivalent to Reno Effluent at Specified Percent

II = Inorganic Nutrients (N and P) Equivalent to Incline Effluent at Specified Percent

Table III presents a summary of the mean maximum growth rates – batch ($\hat{\mu}_b$) of the various source materials tributary to Lake Tahoe on samples at 10 percent concentration with Lake Tahoe water.

TABLE III

MAXIMUM GROWTH RATE – BATCH OF SELECTED SOURCES

Source Sample	Mean Maximum Growth Rate $\hat{\mu}_b$, day^{-1}
Oxidation pond effluents	~ 0.68
Rain	~ 0.56
Septic tank seepage	~ 0.55
Swamp seepage	~ 0.43
Sewage effluent spray seepage . . .	~ 0.41
Surface stream at refuse dump . . .	~ 0.35
Snow melt	0.28

Chemostat–CSTR's

Over fifty laboratory chemostat (continuous flow steady state culture) experiments were conducted to determine the growth response of Selanastrum gracile in four general types of substrate samples. The samples (diluted with Lake Tahoe water) were:

a. surface runoff

b. sewage effluents

c. waste seepage

d. chemical nutrients (nitrogen and phosphorus).

The specific growth rates, μ, were established by selection of residence times in the chemostats to maintain steady-state cultures. The steady-state chemostat growth rates were found to be considerably smaller than the corresponding maximum growth rate, $\hat{\mu}_b$, in the batch cultures. It was not possible under the experimental conditions employed to maintain steady-state growth rates of comparable magnitude to those obtained with batch cultures. That is, washout occurred at these residence times or dilution rates.

For the waste effluents and concentrations of nitrogen and phosphorus in LTW the following assumptions were made as the basis for the M-M kinetic analysis.

a. For samples where the N/P ratio was less than ten, it was assumed that nitrogen (NO_3 + NH_3) was the rate-limiting nutrient.

b. For samples where the N/P ratio was greater than 15, it was assumed that phosphorus (PO_4) was the rate-limiting nutrient.

Available equipment and the time required to conduct a steady-state growth experiment prevented the conduct of parallel batch and chemostat assays on each sample collected. However, considerable effort was devoted to obtaining as much parallel data by the two methods as was possible. Similarly, time did not permit extensive replication of chemostat growth assays to obtain a detailed statistical description of the method. However, when duplicate growth assays on the same sample were conducted, excellent agreement in results was obtained. In addition, kinetic analysis of the effluent growth data in terms of the combined NH_3 and NO_3 concentration, which was the presumed rate-limiting nutrient when the N/P ratio was ≤ 10, yielded excellent correlations for biological systems.

Kinetic Constants

The growth rate response to different sources and concentrations of nutrients is described in terms of the kinetic characteristics of the test organism Selanastrum gracile. These kinetic constants include: the maximum specific growth rate, $\hat{\mu}$, day^{-1}; the yield coefficient, Y, grams of cells produced per gram of nutrient removed; the saturation constant, K_s, $\mu g/\ell$; and, the decay constant, k_d, day^{-1}. Kinetic analysis of the data obtained for sewage effluents, surface runoff, and for inorganic chemical nutrients (when nitrogen is assumed to be limiting, i.e., $N/P \leq 10$) indicated that the maximum specific growth rate, $\hat{\mu}$, had a mean value of 0.196 day^{-1} with 95 percent confidence limits of 0.187 and 0.205 day^{-1}. The small variation in maximum specific growth rates indicates that there is little difference in the biostimulant response produced by the three types of samples when nitrogen is limiting.

The kinetic constants and coefficients found for the three types of samples are summarized in Table IV.

An indication of the adequacy of the M-M kinetic model in describing the growth kinetics of Selanastrum gracile is shown by the apparent fit of the data in Figure 7 (Figures 4-10 LTAC) to the linear transform of the M-M equation. Note that the correlation coefficient was 0.941 which is significant at the 99 percent confidence level.

Several preliminary observations can be made from the data reported in Table IV and Figure 7. First, the level of nitrogen that limits algal growth (at least that of Selanastrum gracile) is extremely low; that is, K_s is less than 15 $\mu g/\ell$. Second, the maximum specific growth rate, $\hat{\mu}$, supported by nitrogen as the limiting nutrient is also quite low – the values ranging from 0.182 to 0.210 day^{-1}. This is excellent agreement for the three types of sample materials ranging from sewage effluents to inorganic laboratory chemicals and additions of nitrogen. Third, a yield coefficient of 6.67 appears quite high; no explanation is available for this observation. Fourth, the much higher growth rates observed for samples where nitrogen was in excess indicates that for most waters (and Selanastrum gracile), nitrogen is not likely to be limiting. In fact it would appear to be difficult, if not impossible, to reduce the level of nitrogen (NH_3 + NO_3) to a level low enough to make it the rate-limiting nutrient.

TABLE IV

SUMMARY OF KINETIC CONSTANTS AND COEFFICIENTS WHEN NITROGEN IS ASSUMED LIMITING

(i.e., $N/P \leq 10$)

Sample → Parameter ↓	Sewage Effluent	Surface Runoff (creeks)	LTW with Nitrogen Added
$\hat{\mu}$ day^{-1}	0.21	0.182	0.192
K_s, $\hat{\mu}$g/ℓ	8.82	2.22	14.4
Y, $\frac{\text{g cells produced}}{\text{g nutrient removed}}$	6.67	-	-
k_d day^{-1}	-0.131	-	-

Similar attempts were made to determine kinetic constants for Selanastrum gracile with samples where phosphate was assumed to be limiting, i.e., ($N/P \geq 15$). However, the correlation of the observed data with the M-M kinetic model was very poor; thus phosphate did not appear to be growth-rate-limiting with any of the samples tested.

Comparison of Batch and Chemostat Assays

One of the surprising results of the parallel batch-chemostat assay studies of samples of surface runoff and waste effluents was the marked difference in the growth rates obtained with the two methods. According to theory, the maximum growth rate in a batch assay with a nutrient concentration S_0 (at start of batch culture growth, t_0) equal to S_1 (the steady-state nutrient concentration in a chemostat), should be markedly less than the steady-state growth rate in the chemostat [i.e., $\hat{\mu}_b < \mu$ when S_0 (batch) = S_1 (chemostat)]. However, exactly the opposite was observed as is shown in Figure 8. The maximum growth rates in batch culture, $\hat{\mu}_b$, for sewage effluents or with inorganic nutrients (N and P) added to LTW in equivalent concentration were found to be about four times greater than the specific growth rates observed in steady state — continuous flow cultures. From these data it appears that the batch type of laboratory growth assays gives spurious results — or at least results that cannot be maintained at steady state. Possibly this pulse growth response results from the initial storage or carry-over of nutrients in the cell from the stock culture. An adequate explanation for this phenomenon has yet to be developed.

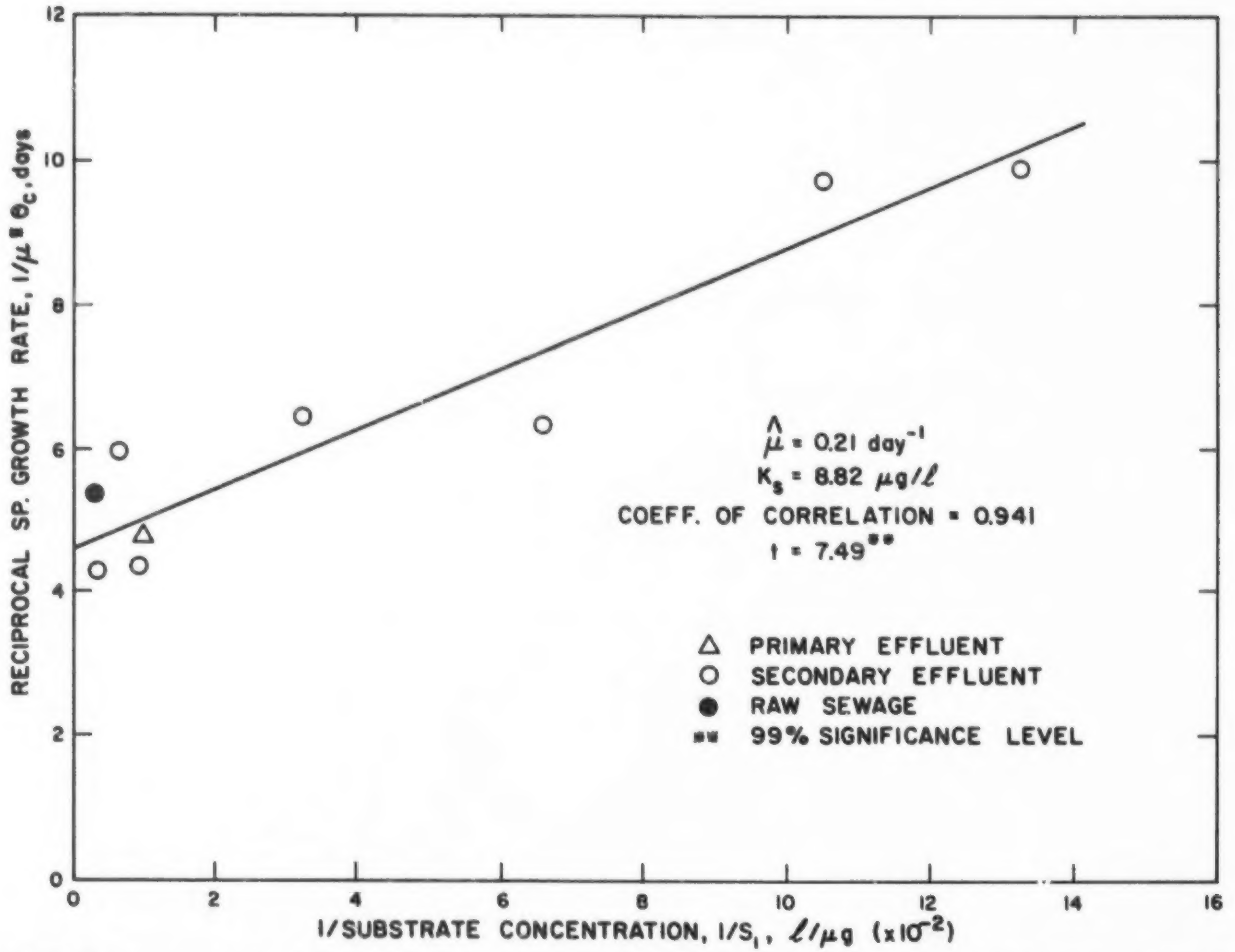


FIGURE 7. RECIPROCAL GROWTH RATE – ASSUMED SUBSTRATE-LIMITING CONCENTRATION RELATIONSHIP FOR NITROGEN IN SEWAGE EFFLUENTS

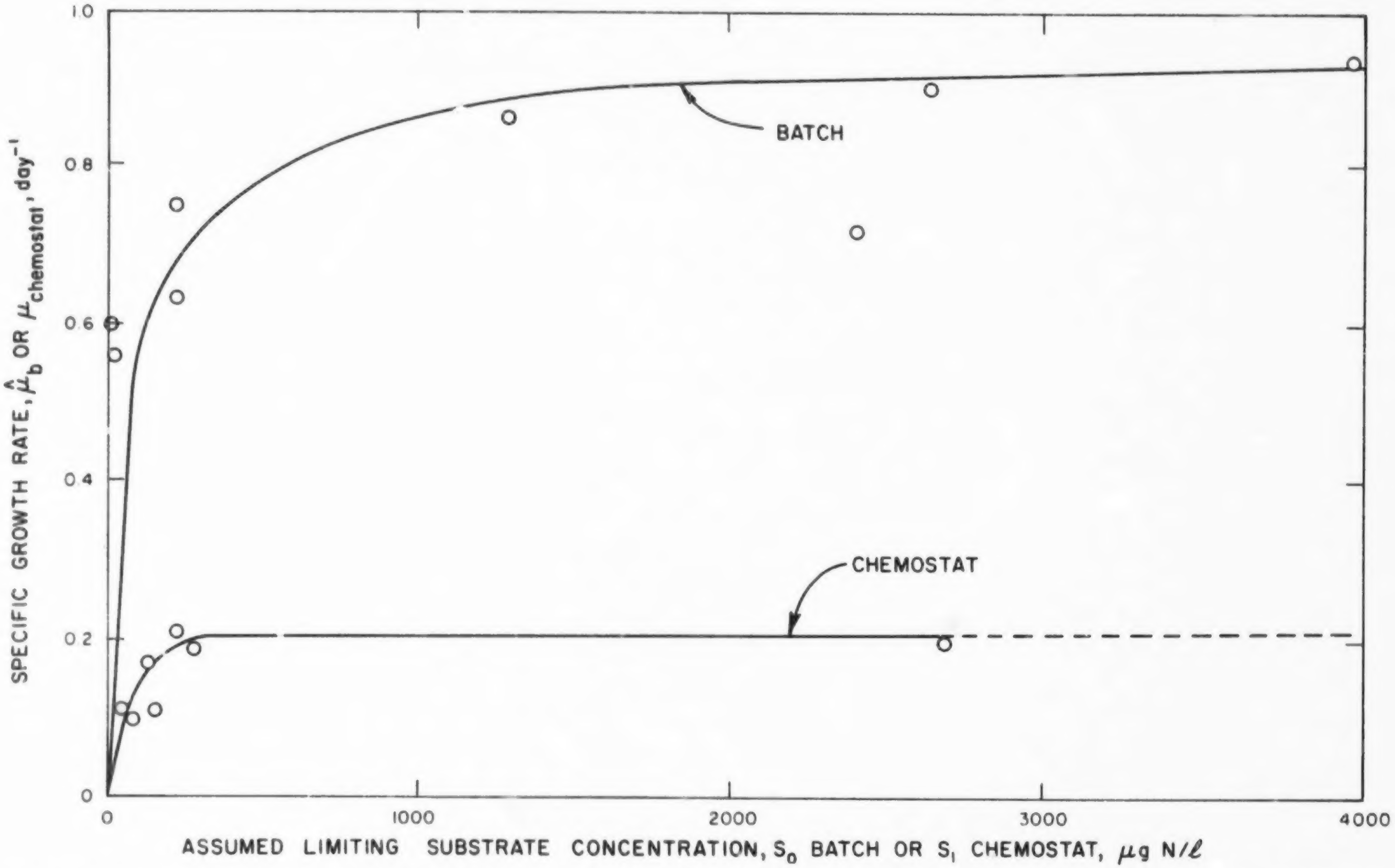


FIGURE 8. COMPARISON OF BATCH AND CHEMOSTAT GROWTH RATES FOR ASSUMED NITROGEN-LIMITING CONDITIONS WITH SEWAGE EFFLUENTS

To explore further the finding that the maximum growth rates, $\hat{\mu}_b$, obtained from batch cultures were greater than the corresponding steady-state growth rates (i.e., when S_o batch = S_1 chemostat), an analysis was made of the initial or transient growth rates obtained after inoculating a chemostat with the test alga and following the growth rate until steady-state cell concentrations were reached. This is equivalent to making a batch-type growth rate analysis of a chemostat during the period that the culture was approaching a steady state. It was surprising to find that the initial transient growth rate in the chemostat unit was even greater than the maximum growth rate, $\hat{\mu}_b$, in the batch assay. In fact, an analysis of 25 pairs of $\hat{\mu}_b$ and $\hat{\mu}_{transient}$ $(\hat{\mu}_{tr})$ indicates that the maximum transient growth rate was correlated roughly with the batch assay maximum growth rate as follow :

$$\hat{\mu}_{tr} = 1.78\ \hat{\mu}_b \quad .$$

From these data, it appears that the maximum growth rates in the batch-type assay, as well as the initial transient growth rates in a chemostat, are not at all representative of the growth rates that can be maintained in a near steady-state culture system. Additional investigation of this anomaly of algal culture systems is necessary.

CONCLUSIONS

The growth rate of the test alga Selanastrum gracile in Lake Tahoe water (LTW) at 20°C was found to average about 0.29 day^{-1}. However, there was a marked seasonal difference in the enrichment of LTW. The average growth rate for samples collected in late summer and early winter was 0.16 day^{-1} and the average for samples collected in the spring (April) was 0.430 day^{-1}. These differences were significant statistically at a confidence level greater than 95 percent.

Of the four creeks sampled, namely, Meeks, Ward, Incline, and Truckee-Trout, only Truckee-Trout yielded higher mean growth rates, $\hat{\mu}_b$, (statistically significant at greater than 95 percent confidence limit) than the remainder of the creeks at all of the sample concentrations tested (i.e., 1%, 10%, and 50% samples in LTW). The only other significantly higher growth rate ($\hat{\mu}_b$) was observed for the 50 percent concentration sample from Incline Creek.

The mean maximum growth rate ($\hat{\mu}_b$) of all waste effluents was found to be 0.66 day^{-1} with a range of from 0.48 day^{-1} for oxidation pond effluents to about 0.78 day^{-1} for primary and secondary effluents. All effluents reported higher values of $\hat{\mu}_b$ than those observed for raw sewage at the 1 percent and 10 percent concentration levels.

The observed mean growth rates, $\hat{\mu}_b$, for effluent samples were generally significantly higher than that resulting from equivalent concentrations of nitrogen or of nitrogen and phosphorus in LTW. Moreover, the maximum cell concentration, $\hat{X}$, of the effluent samples averaged 500 cells/cu mm greater than that of the equivalent concentration of nitrogen and phosphorus in LTW.

In about 60 comparative series of assays, nitrogen (NO_3 and NH_3) was added to LTW in concentrations equivalent to that in the field sample tested, and there was no significant difference between the maximum growth rate of samples of LTW that had equivalent amounts of nitrogen (NO_3 and NH_3) alone ($\hat{\mu}_b = 0.57$ day^{-1}) and phosphorus and nitrogen in combination ($\hat{\mu}_b = 0.61$ day^{-1}). Phosphorus alone added to LTW did not increase the growth rate significantly. These data indicate that LTW is not affected significantly by the addition of substantial amounts of phosphorus. Lake Tahoe as well as a majority of the inflows to Lake Tahoe were found to be nitrogen limiting. This might be expected considering the fact that the average ratio of nitrogen (NH_3, NO_2, and NO_3) to phosphorus (soluble reactive P) in all of the inflow samples tested was 2.8.

According to kinetic theory, the maximum growth rate in a batch assay with a nutrient concentration S_o (at $t = 0$) equal to S_1 (steady-state nutrient concentration in the effluent from a chemostat) should be markedly less than the steady-state growth rate in the chemostat [i.e., $\hat{\mu}_b < \mu$ when S_o (batch) $= S_1$ (chemostat)]. However, exactly the opposite was observed in this study. The $\hat{\mu}_b$ for sewage effluents or with inorganic nutrients (N and P) added to LTW in equivalent concentrations was found to be about four times greater than the specific growth rate observed in steady state — continuous flow cultures.

To evaluate the unusual results obtained with batch and continuous flow cultures, the initial growth rates, $\hat{\mu}_{transient}\left(\hat{\mu}_{tr}\right)$, were evaluated from the chemostat results during the period that the chemostat was approaching steady state. An approximate correlation between $\hat{\mu}_{tr}$ and $\hat{\mu}_b$ was found to be as follows:

$$\hat{\mu}_{tr} \cong 1.8\ \hat{\mu}_b \quad .$$

It appears that with batch-type assay data, initial growth response of the test alga is considerably greater than the growth rate that can be maintained under steady-state conditions. Additional study of this anomaly of algal culture systems is necessary.

Preliminary evidence from both batch and continuous flow assays indicates that the biostimulatory properties of secondary effluents are considerably greater than that of raw or primary treated wastes. Additional comparative studies of the three states of the same waste are needed with sufficient replication to show whether the apparent differences are statistically significant.

Sewage effluents of all types exhibited consistently higher growth response rates than could be accounted for by the amount of nitrogen and phosphorus present in the samples. For example, continuous flow growth assays of waste effluents and equivalent inorganic nutrients yielded M-M maximum growth rate constants, $\hat{\mu}$, of 0.18 and 0.19 day^{-1} at infinite nitrogen (NH_3 and NO_3) concentration. However, specific growth rates as high as 0.21 to 0.24 day^{-1} were observed for sewage effluent samples with nitrogen concentrations (in excess) in the range of 0.1 to 0.2 mg/ℓ as N.

The results of this investigation have raised serious question as to both the sensitivity and general applicability of the batch-type assay in quantitating the growth characteristics of nutrients and waste materials, especially at low concentrations of nutrients. The gross lack of sensitivity of the batch assay was well demonstrated in the assessment of the biostimulatory properties of four creeks tributary to Lake Tahoe. The applicability and credibility of the batch assay was seriously questioned by the unusual results obtained (contrary to theory) with parallel studies of the batch and continuous flow assay systems. Additional work is necessary to clarify or confirm both of these questionable characteristics of the batch assay and to further verify the general applicability of continuous flow kinetic characterization of nutrient and waste materials.

ACKNOWLEDGMENTS

The study was made possible by a Demonstration Grant [WPD 48-01 (R1)] from the Federal Water Pollution Control Administration to the Lake Tahoe Area Council (LTAC). Technical direction of the work was provided by Professors P. H. McGauhey, G. A. Rohlich, and E. A. Pearson of the Lake Tahoe Area Council Board of Consultants. Dr. Charles E. Goldman, University of California, Davis, and Dr. Arthur D. Hasler, University of Wisconsin, served as special consultants to the LTAC Board of Consultants. Laboratory facilities, outdoor space for pilot ponds, and facilities for staff were developed at Tahoe City through cooperation of the California Department of Fish and Game which maintains a hatchery building at that location. Cooperating with the Lake Tahoe Area Council and its consultants in the conduct of the demonstration program were the following organizations: California Regional Water Quality Control Board No. 6; Nevada State Department of Public Health; California State Department of Public Health; California State Department of Fish and Game; U. S. Public Health Service, Region IX; Federal Water Pollution Control Administration; California State Water Quality Control Board; Douglas County Health Department; and the Placer County Health Department.

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EFFECT OF SURFACE/VOLUME RELATIONSHIP, CO_2 ADDITION, AERATION, AND MIXING ON NITRATE UTILIZATION BY *Scenedesmus* CULTURES IN SUBSURFACE AGRICULTURAL WASTE WATERS

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INTRODUCTION

At Firebaugh, California, an interagency group (U. S. Bureau of Reclamation, U. S. Federal Water Pollution Control Administration, and the California Department of Water Resources) is investigating methods of removing nitrate-nitrogen from subsurface agricultural drainage water. One of the methods being studied is algae stripping, a process in which the growth of algae is encouraged, the nitrate is converted to cellular materials, and then the algae is separated from the liquid phase by centrifugation, flocculation, etc. We are using algae of the genus *Scenedesmus* (originally obtained from a high-rate sewage pond at the University of California's Richmond Field Station) in our system. Although the drainage water is high in dissolved salts (TDS 5000-10,000 mg/ℓ), it is not a complete media for the growth of *Scenedesmus*. We have been conducting a number of laboratory studies to determine the nutrient additions necessary for optimum growth (and nitrogen removal) of the algae in our water. Some questions have been raised on the correct (and the most reliable) experimental procedures to follow in these algal assay experiments.

One experiment which might be of interest to workers in the field of algae assays was recently conducted to determine the effect of flask size, surface/volume ratio, and type of mixing (including aeration and the addition of carbon dioxide). The test was performed at the Firebaugh Treatment Site and the Department of Water Resources' laboratory at Bryte, California. Because neither of the participating laboratories had the capacity to accomplish all of the mixing parameters, the study had to be conducted at both the Firebaugh and Bryte laboratories. In general, an attempt was made to reduce the number of variables between the two laboratories. The same culture media, inoculum, type of flask, and duration of lighting were used at each facility. However, there was considerable variation in both temperature and light availability. Bryte worked with constant stirring and hand swirling and Firebaugh concentrated on aeration, carbon dioxide addition, and hand swirling. The experiment was conducted during the period May 15 through May 21, 1969.

METHODS

Water

The water used as a growth media at Firebaugh Center was subsurface drainage (from irrigation water applied to fields above the tile drain) and was relatively high in total dissolved solids. Water quality varied from field to field and seasonally, depending on the type of crop being grown. Water for the algae project came from a field that had been planted in rice for the past three seasons. With the increased flow from the rice paddies, there was considerable fluctuation in dissolved salts from season to season. Table I shows a typical comparison of chemical analyses completed before and during flooding of the rice field.

TABLE I

CONCENTRATION OF MINERAL CONSTITUENTS IN TILE DRAIN WATER
FROM SUMP AT FIREBAUGH
(mg/ℓ)

Constituent	Date	
	5-2-67	7-14-67
Calcium	373	194
Magnesium	190	106
Sodium	1390	875
Potassium	5.4	4.1
Carbonate	29	0
Bicarbonate	247	373
Sulfate	3600	2070
Chloride	559	304
Nitrate-N	17.6	9
Hardness	1710	921
Total Dissolved Solids	6590	3950
Boron	14	9.3

The quality of the experimental water for this study was comparable to the May 12 data shown in the preceding table. Nitrate-nitrogen was about 21 mg/ℓ during this study and additional nutrients, based on the results of previous studies, were added. The nutrient additions were PO_4-P, 3 mg/ℓ (added as H_3PO_4), and Fe, 3 mg/ℓ (added as $FeCl_3$). The original pH of the water was 7.6 and the total alkalinity was approximately 340 mg/ℓ as $CaCO_3$.

Inoculum

To obtain a somewhat standard inoculum that was predominantly Scenedesmus, we used the 1/4-acre high-rate pond for a source of algae. This pond is well mixed, on a ten-day hydraulic detention time, and has maintained a population of approximately 350,000 4-celled Scenedesmus quadricauda per milliliter for the past few months. Total numbers of other species present is less than 10,000 cells per milliliter. Inocula for our laboratory cultures is collected from surface samples since the young, actively growing algal cells tend to remain in the upper water levels.

The inoculum for this study was collected from the high-rate pond and was used both at Firebaugh and Bryte laboratories. The algae were kept refrigerated in the dark while being transported from Firebaugh to Bryte (near Sacramento). All flasks were individually inoculated with approximately 3000 cells per milliliter.

Light Box Facilities

Firebaugh: The light box at Firebaugh is built inside a converted office trailer and consists of two shelves, 12 x 3 ft. Lighting is from eight 6-ft, cool-white, fluorescent bulbs placed 15 in. above the surface of the shelf. Light intensities at the shelf are approximately 350-400 ft-c. All ballasts were removed from the light fixtures and placed several feet from the light box to minimize temperature increases. The lights were connected to a timer, but in this study the lighting was continuous and external light sources were excluded.

In the study, aeration with compressed air and compressed air enriched with 4 percent CO_2 were tested. A small compressor provided the air which was passed through two water scrubbers before being distributed to the growth flasks through a central manifold. The enriched air was bottled with 4 percent CO_2 and was passed through a water scrubber for humidification (to reduce evaporation losses in the culture containers). Gas volume was regulated by placing a short section of capillary tubing in the air line between the manifold and the flask.

During the present study, the light box at Firebaugh was not provided with an adequate method of temperature control; consequently, air temperatures exhibited daily variation of as much as 8°C. Although temperatures were not controlled to the desired degree, the temperature fluctuation did not appear to have an adverse effect on the algae, and the fluctuations were little more than the algae encountered in the shallow outside pools.

Bryte

The Bryte Laboratory acquired a light box originally built by the California Department of Fish and Game personnel at Stockton. In this box, the flasks were stirred by means of a series of belt and pulley-driven glass rods. Temperature was maintained at 24°C by means of a recirculating water bath. Continuous lighting was also provided by cool-white fluorescent bulbs placed beneath clear glass shelves.

Experimental Design

The experiment was designed to determine the effect of different flask sizes (250, 500, and 1000 mℓ), different surface/volume ratios (flasks filled to either one-half or full volume), and various means of mixing and/or aeration (swirled twice daily, 4 percent CO_2 injection, atmospheric air injection, and continuous stirring). The design is outlined in Figure 1.

Observations

On the first day, chemical and biological observations were made at both laboratories. These measurements included: 1) NO_3-N (brucine only at Bryte, and at Firebaugh specific ion electrode determinations with random brucine analyses to establish the standard curve for the electrode); 2) spectrophotometer absorbance at 410 mμ; 3) volatile suspended solids; 4) cell counts and species determination; 5) temperature; 6) pH; and 7) *in vivo* fluorescence (Bryte only).

During the study, nitrates were run daily at Firebaugh (electrode and brucine) and every other day at Bryte (brucine only). Absorbance and fluorescence (Bryte) readings were determined daily. Volatile solids, cell counts, and species composition were determined at the end of the experiment. All flasks were shaken thoroughly before subsampling.

RESULTS

In Figures 2 through 4 plots of the nitrate remaining versus time data are presented, which show the effect of different methods (CO_2 injection, aeration, flask size, volume, etc.) on nitrogen uptake by *Scenedesmus* cultures at Firebaugh. In those flasks with CO_2 injection the effect of the surface area/volume relationship was not too noticeable as all cultures were at or near zero nitrate at the end of 4 days. With aeration by compressed air, the effect of surface area became more apparent. The most rapid rate of nitrate removal occurred in either 250 or 500-mℓ flasks filled to one-half capacity and in all instances removal was more rapid in the partially filled flasks. In the CO_2 flasks nitrate nitrogen was reduced from more than 20 mg/ℓ to zero in less than 4 days. The rate of removal was slightly slower in the aerated flasks (4-5 days), as compared to CO_2 addition.

The effect of surface area/volume was even more apparent in those cultures that were swirled only. Again the best flasks were 250 mℓ with 125 mℓ of culture (zero nitrate at 6 days) and in all instances the partially filled flasks were better than those filled to capacity. At the end of 6 days only the cultures in the 250-mℓ flasks with 125 mℓ volume had removed all of the nitrogen.

In those cultures grown at Bryte Laboratory, the same general trends were noted (Figures 5 and 6). Again the 250-mℓ flasks with 125 mℓ of culture were the most rapid and the completely filled 1,000 mℓ flasks were the slowest. Intermediate surface area/volume effects were not as discrete as at Firebaugh. One interesting result of the Bryte studies was the apparent similarity between flasks swirled twice daily and those cultures that were continuously mixed at 80 rpm by rotating glass stirring rods. Also a comparison of swirled flasks at Bryte and Firebaugh indicates that the growth characteristics of cultures at both laboratories were similar, in spite of differences in lighting and temperature control.

<table>
<tr><td colspan="2" rowspan="4"></td><td colspan="6">FLASK SIZE (mℓ)</td></tr>
<tr><td colspan="2">250</td><td colspan="2">500</td><td colspan="2">1000</td></tr>
<tr><td colspan="6">CULTURE VOLUME (mℓ)</td></tr>
<tr><td>125</td><td>250</td><td>250</td><td>500</td><td>500</td><td>1000</td></tr>
<tr><td rowspan="5">TYPE OF AERATION</td><td rowspan="3">FIREBAUGH LAB</td><td colspan="6">SWIRLED 2x / DAY</td></tr>
<tr><td colspan="6">4% CO_2</td></tr>
<tr><td colspan="6">ATM. AIR</td></tr>
<tr><td rowspan="2">BRYTE LAB</td><td colspan="6">SWIRLED 2x/DAY</td></tr>
<tr><td colspan="6">CONTINUOUS SWIRL</td></tr>
<tr><td colspan="8">3 REPLICATIONS PER COMBINATION OF VARIABLES</td></tr>
</table>

FIGURE I. EXPERIMENTAL DESIGN USED IN THE FIREBAUGH AND BRYTE STUDY

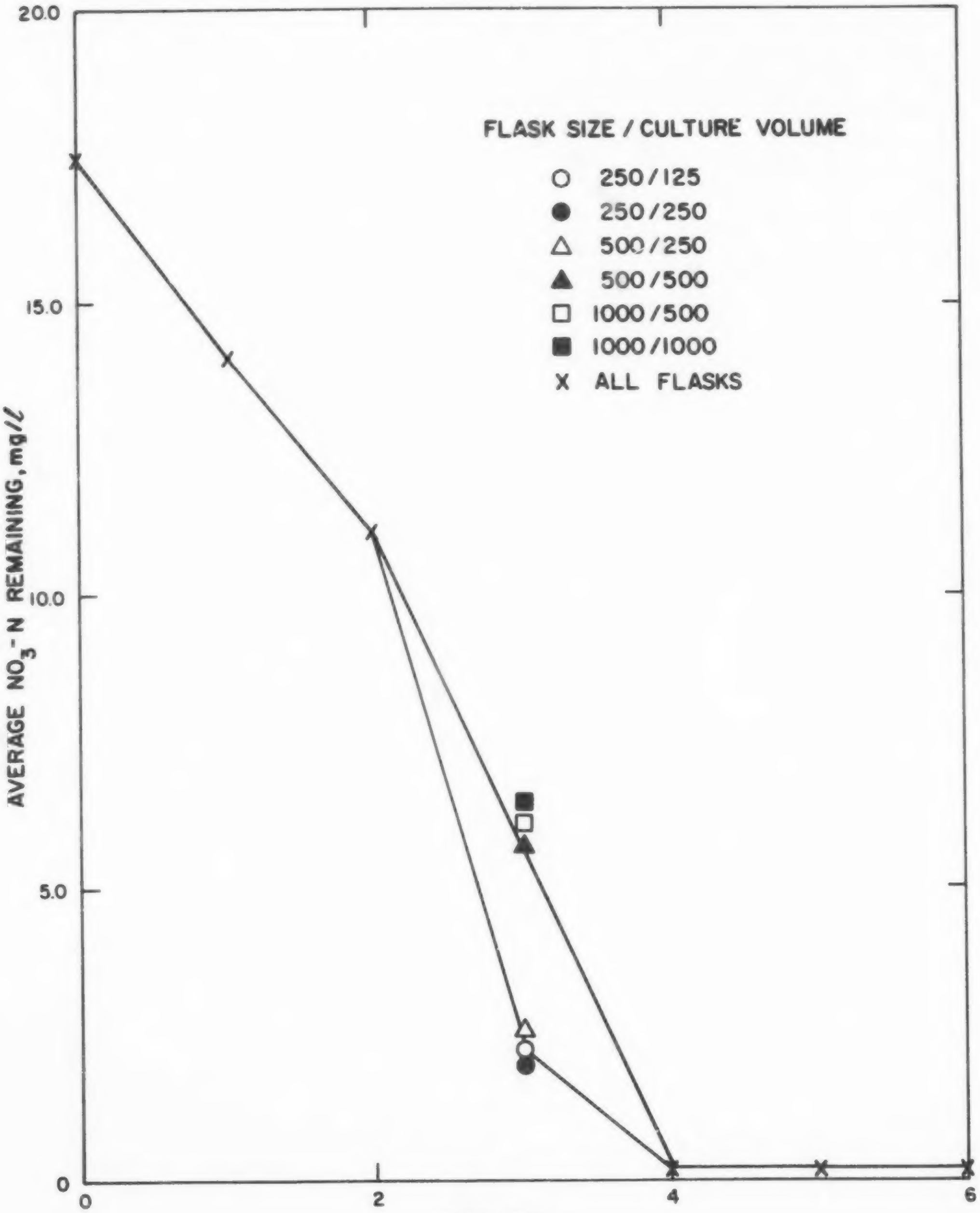

FIGURE 2. NITRATE UTILIZATION BY *Scenedesmus* CULTURES AERATED WITH 4% CO_2 IN COMPRESSED AIR

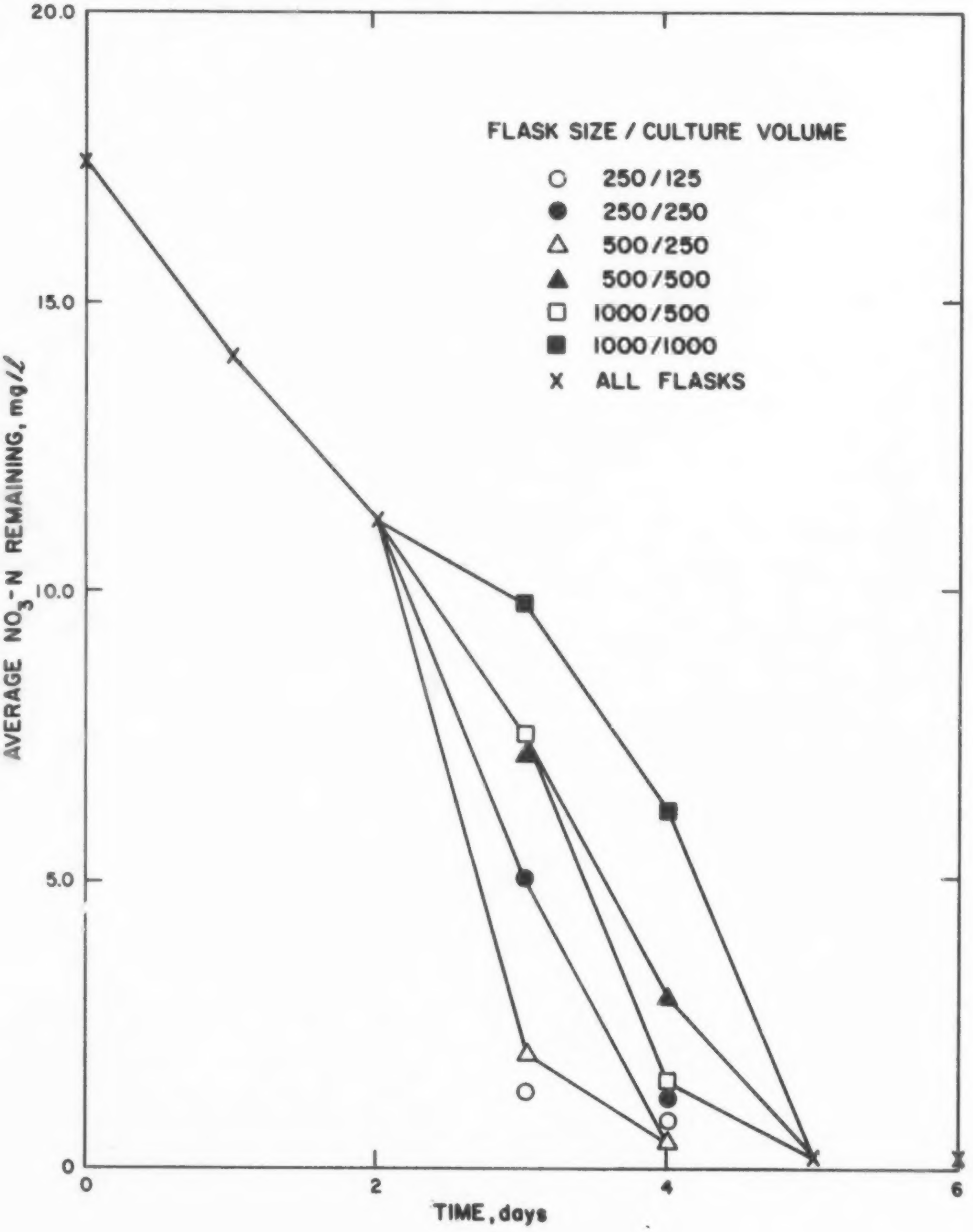

FIGURE 3. NITRATE UTILIZATION BY *Scenedesmus* CULTURES AERATED WITH COMPRESSED AIR AT FIREBAUGH, AVERAGE OF THREE REPLICATIONS

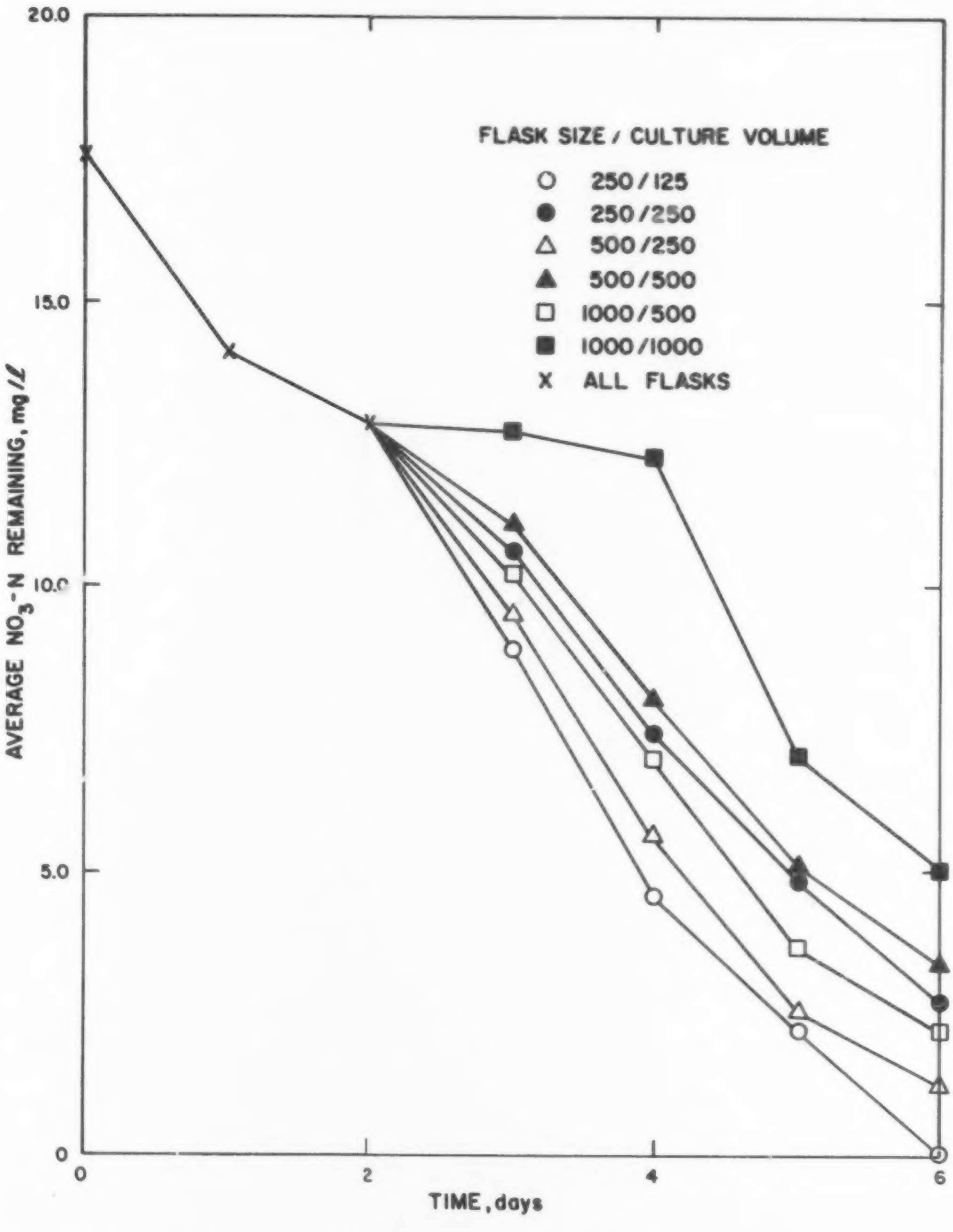

FIGURE 4. NITRATE UTILIZATION BY *Scenedesmus* CULTURES SWIRLED ONLY, FIREBAUGH LABORATORY, AVERAGE OF 3 REPLICATIONS

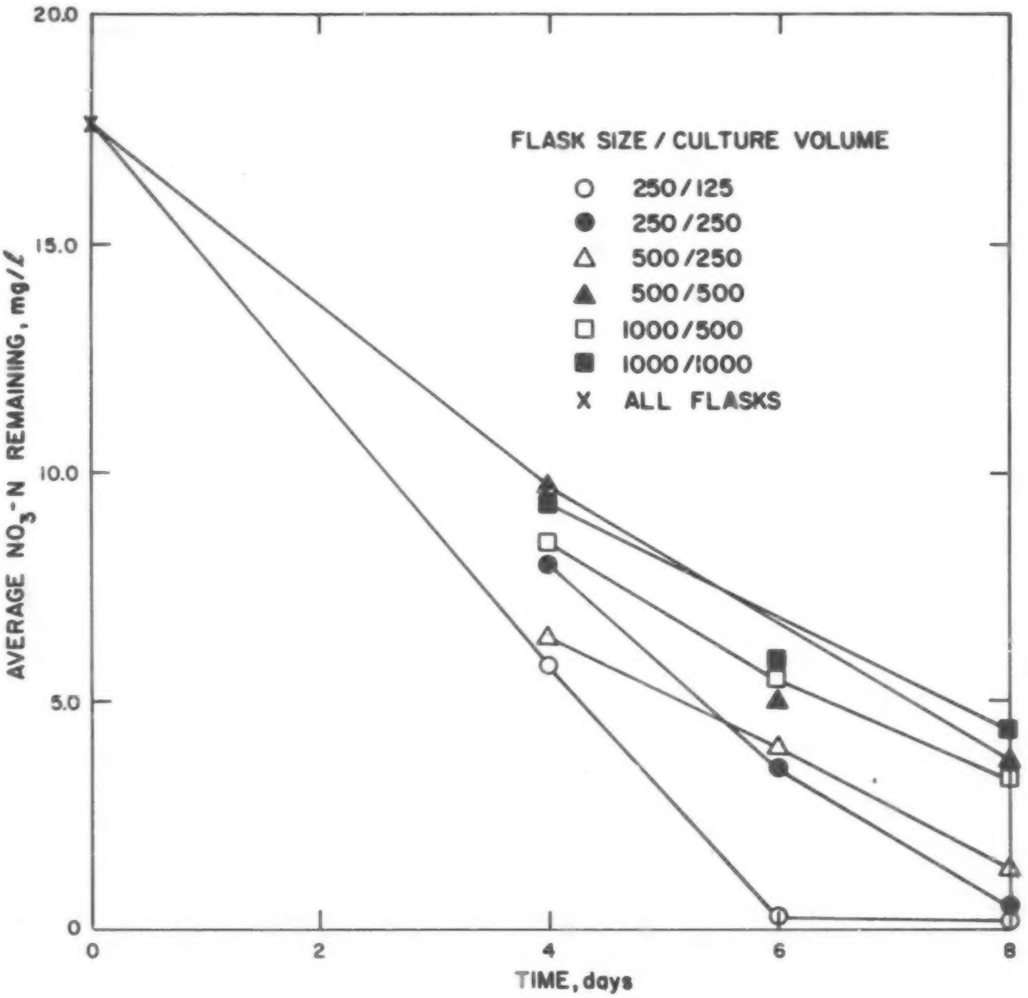

FIGURE 5. NITRATE UTILIZATION BY *Scenedesmus* CULTURES SWIRLED ONLY, BRYTE LABORATORY, AVERAGE OF 3 REPLICATIONS

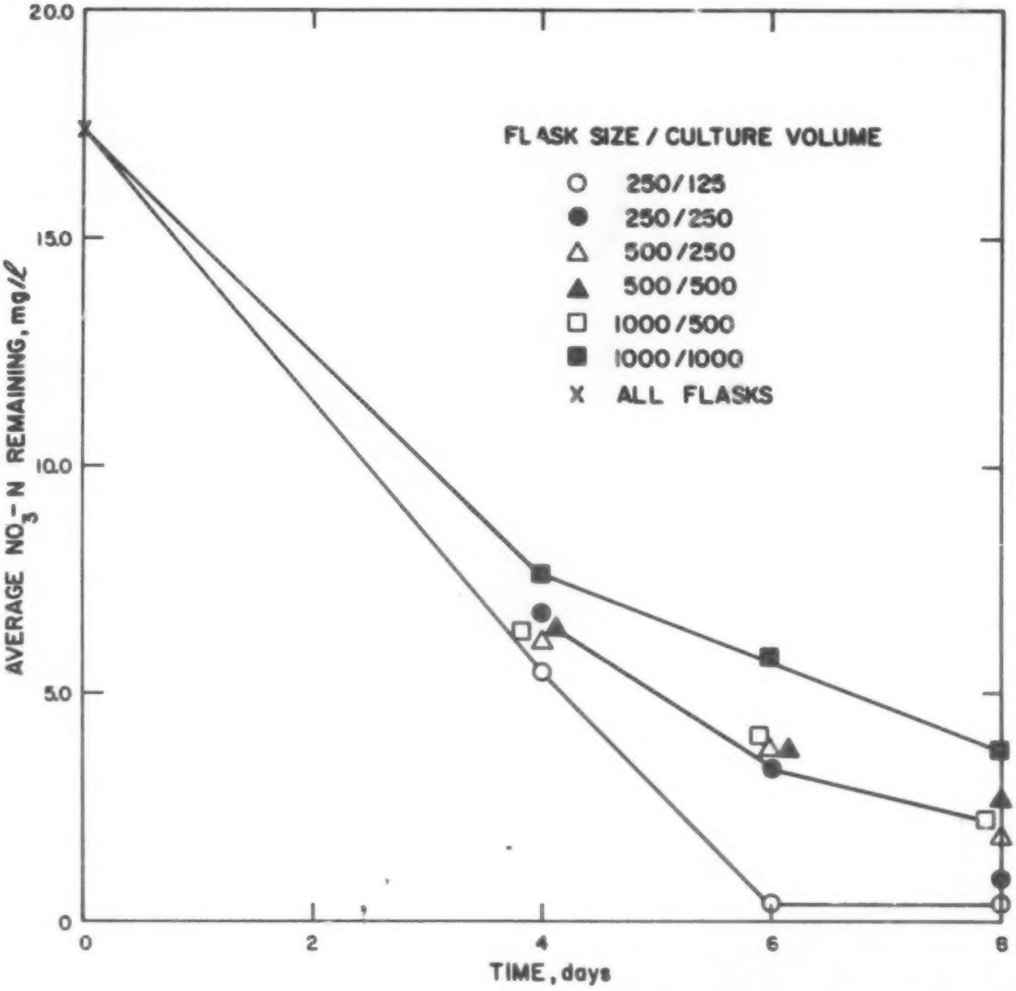

FIGURE 6. NITRATE UTILIZATION BY *Scenedesmus* CULTURES CONTINUOUSLY STIRRED AT BRYTE LABORATORY, AVERAGE OF 3 REPLICATIONS

Another way of looking at the data is to compare average suspended volatile solids remaining at the end of the run (Figure 7). The highest volatile solids are in the smallest flask filled to one-half capacity. The lower volatile solids in those flasks enriched with CO_2, as compared to aeration with compressed air, may be because the cultures were nitrogen starved for the last three days of the study and may have undergone some bacterial decomposition. In general, the filled containers had the lowest biomass, although the filled 250-mℓ aerated flask had an unusually high biomass, possibly an analytical error. Absorbance values (at 410 mμ) show the same trend as volatile solids.

Cell numbers (Figure 8) varied with the culture conditions. Terminal counts showed that in the very active cultures, small volumes in small flasks, the organisms were quite numerous and predominantly (more than 90 percent) single-celled. Examination of the cells indicated that the cultures with the most rapid growth rate were predominantly very small, single-celled Scenedesmus. This contrasted with the filled flasks which had fewer and larger cells of mostly 2- and 4-celled coenobia. The inoculum was of the 4-celled stage.

In vivo fluorescence which was measured at the Bryte Laboratory has been compared to NO_3-N remaining (Figure 9) and appears to be a sensitive indicator of algal growth. Generally, fluorescence units corresponded inversely to NO_3-N remaining and directly to volatile solids and absorbance (Figure 10).

DISCUSSION

The effect of flask size and volume (surface-to-volume ratio) was as we expected and has been reported in the literature (Brock 1966). Maximum growth or nitrogen removal always occurred in the 250-mℓ flasks filled with 125 mℓ of culture and the remaining partially filled flasks were better than the filled flasks. Based on these data, the smaller flasks would seem the more desirable. In practicality, we feel that 500-mℓ flasks with 250 to 300 mℓ of culture are more usable. With this combination there is an adequate amount of culture to allow for fairly extensive analytical measurement.

At Firebaugh we have made aeration with compressed air the standard way of mixing algal cultures. The data indicated that compressed air alone effected almost the same rate of growth as compressed air enriched with CO_2 and was much more economical and easier to handle. The enriched air was purchased in cylinders which had to be changed daily and returned to the vendor.

Both CO_2 addition and aeration appeared to be affecting the algal growth rate, but not the ultimate yield. In the agricultural drainage water (bicarbonate and total alkalinity of 340 mg/ℓ as $CaCO_3$) used at Firebaugh, carbon was probably a limiting element and the rate of exchange across the surface of the culture was not rapid enough with swirling only. This might explain why the stirring apparatus at Bryte was little or no better than hand swirling with respect to nitrogen removal. The stirring rods kept the algae in suspension quite well but did not bring more water in contact with the atmosphere.

Addition of CO_2 at the rates used at Firebaugh caused the pH to remain at the 7.3 to 7.6 range, as compared to 9.8 to 10.5 in the remaining series of flasks. This pH difference caused changes in nutrient availability between the various systems. In another experiment at Firebaugh to determine the optimum amount of iron and phosphorus required to remove nitrogen, the type of mixing

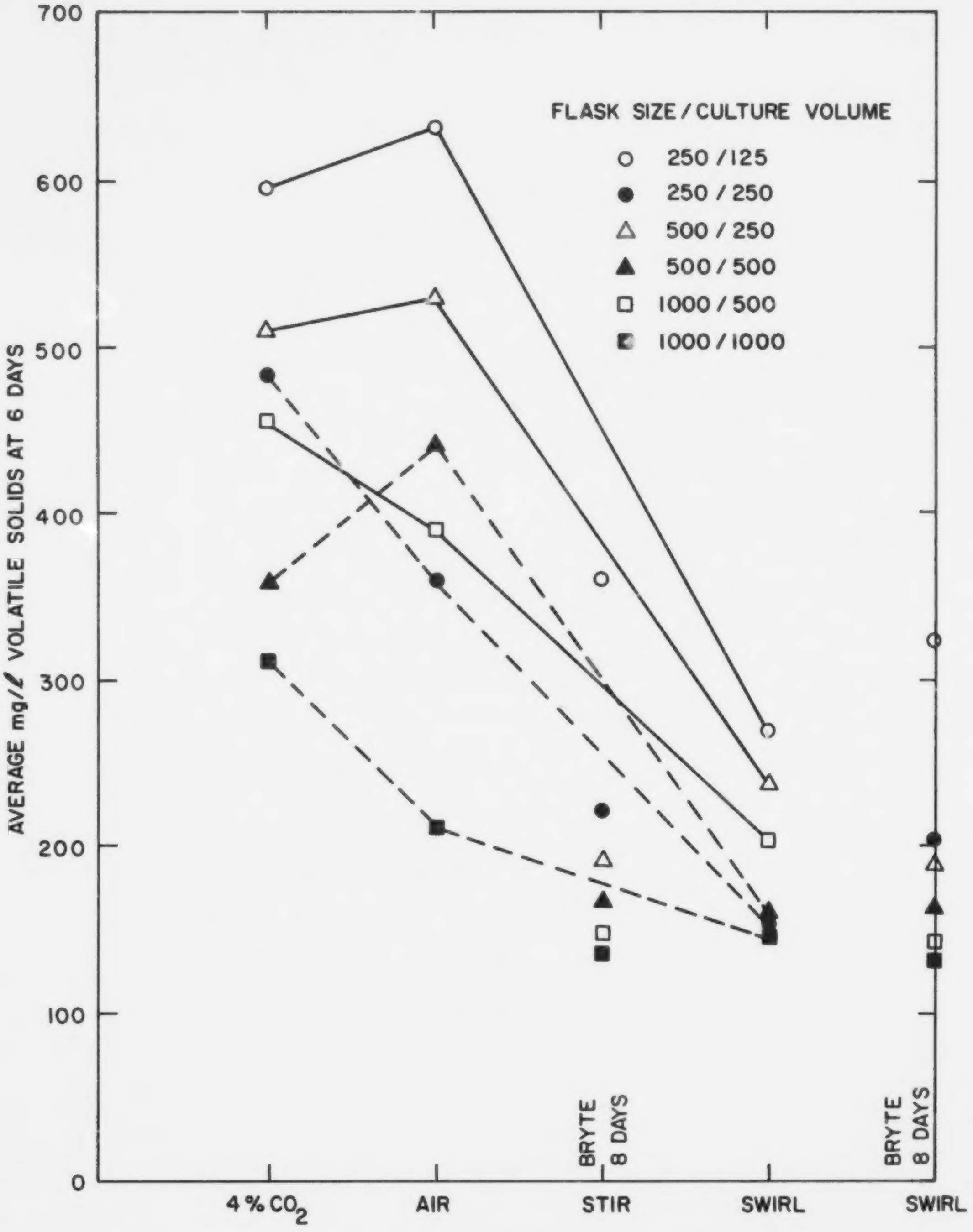

FIGURE 7. EFFECT OF SURFACE/VOLUME RATIO, AERATION, CARBON DIOXIDE INJECTION AND MIXING ON VOLATILE SOLIDS AT SIX DAYS, AVERAGE OF 3 REPLICATIONS

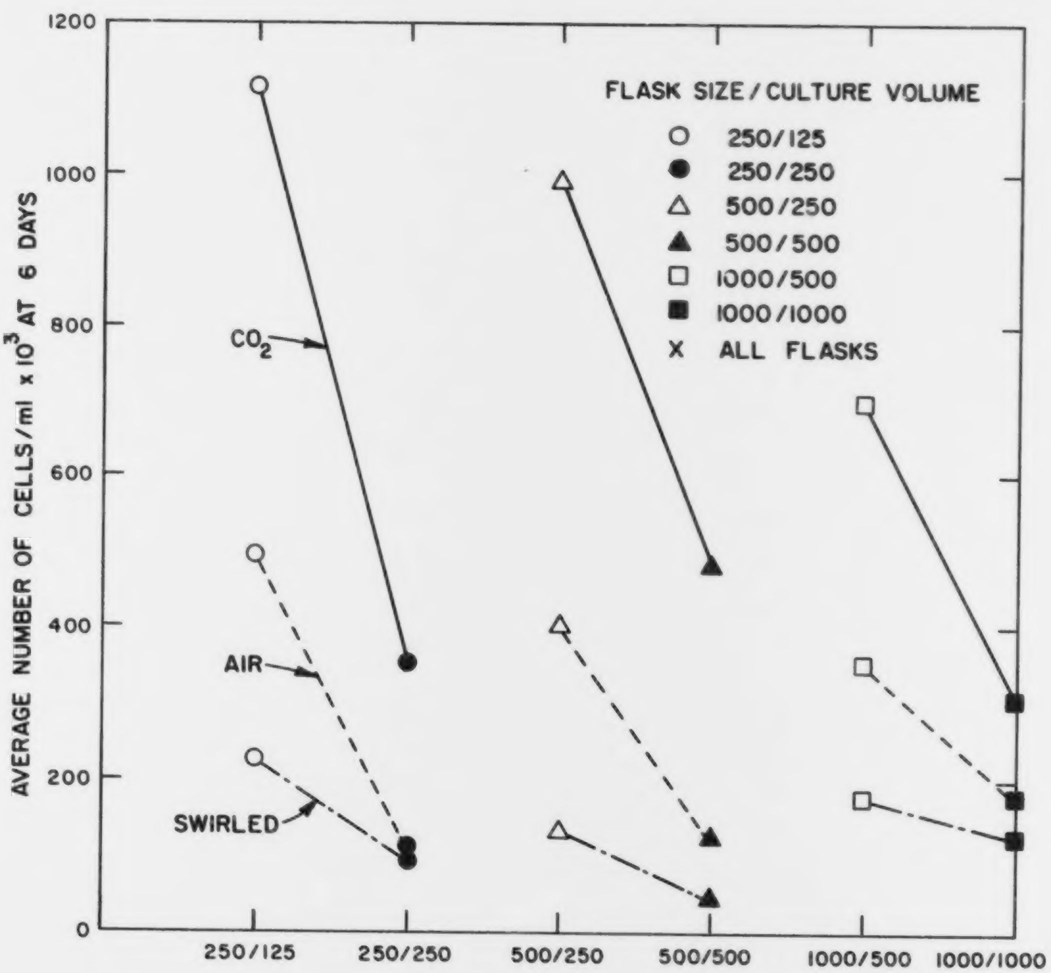

FIGURE 8. EFFECT OF SURFACE/VOLUME RELATIONSHIP AND VARIOUS TYPES OF AERATION ON CELL NUMBERS AT SIX DAYS, AVERAGE OF 5 REPLICATIONS

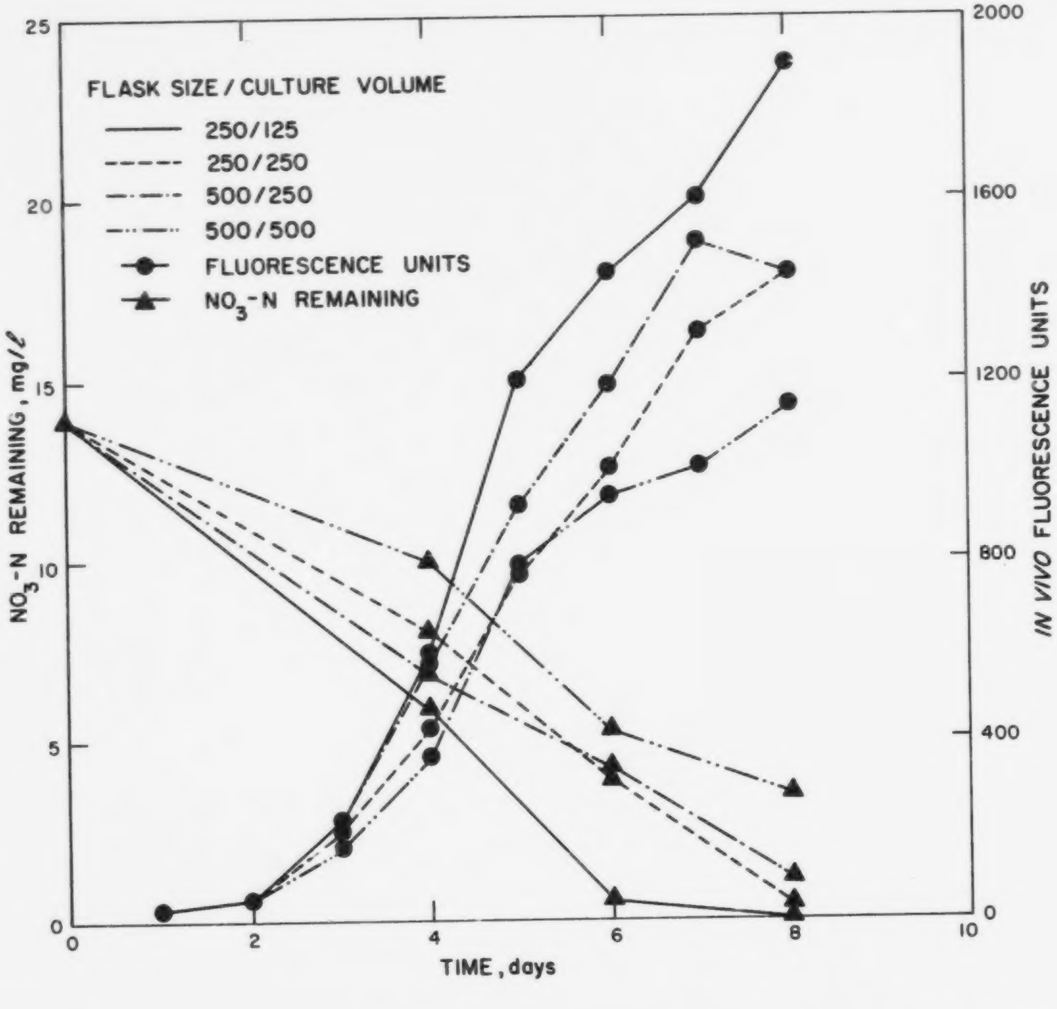

FIGURE 9. FLUORESCENCE AND NITRATE-NITROGEN REMAINING vs. TIME FOR VARIOUS SIZES OF SWIRLED FLASKS AT BRYTE LABORATORY

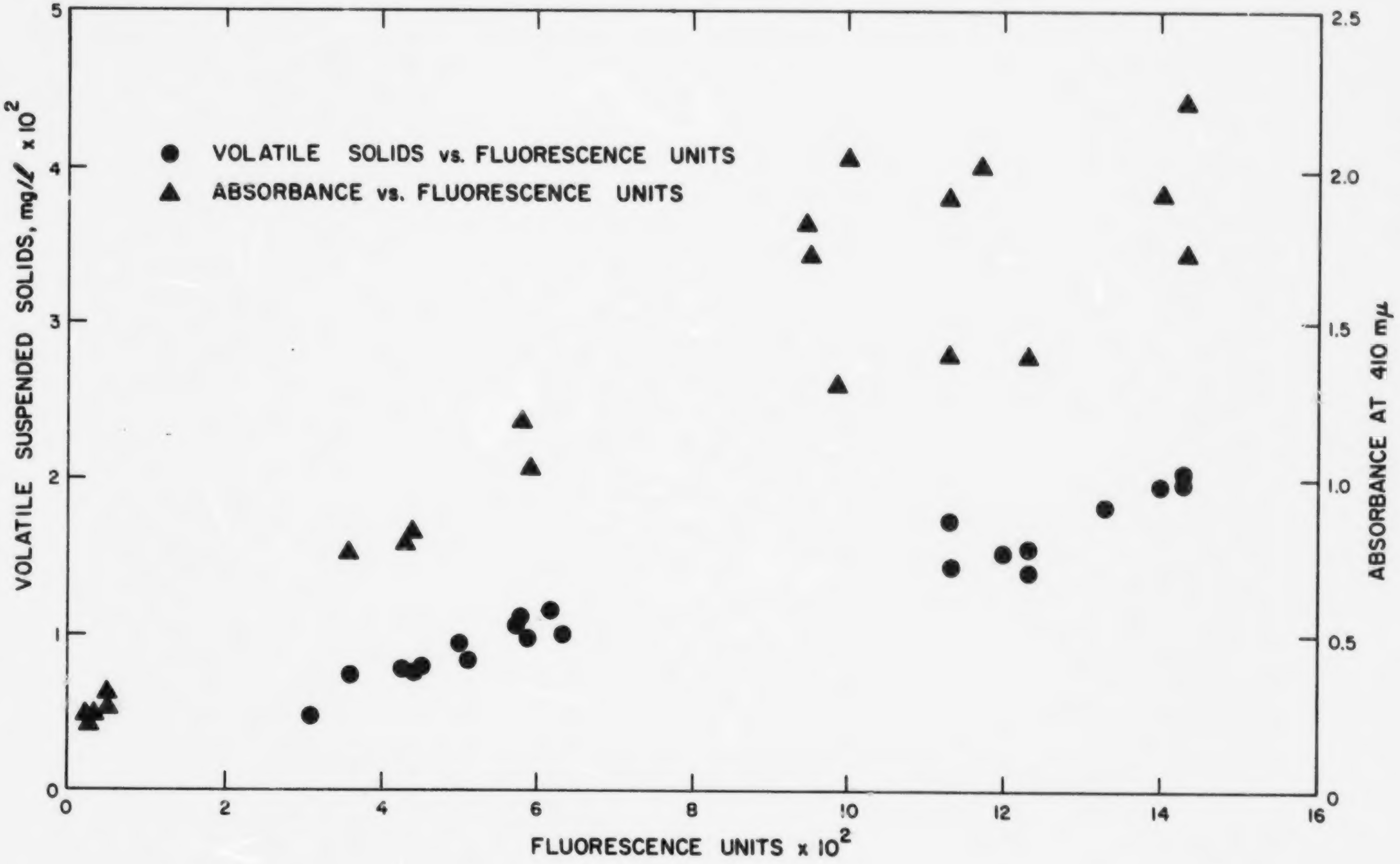

FIGURE 10. COMPARISON OF FLUORESCENCE VALUES WITH VOLATILE SOLIDS AND ABSORBANCE AT BRYTE LABORATORY, ALL POINTS ARE AVERAGE OF 3 REPLICATIONS

also had a significant effect (Figure 11). At 0 iron addition, and 2 mg/ℓ P (added as H_3PO_4), NO_3-N remaining at 16 days (continuous lighting) ranged from about 2 mg/ℓ to 9 mg/ℓ , to 13 mg/ℓ, depending on whether the flasks respectively received CO_2, compressed air, or were swirled. Evidently at a pH of 7.3 to 7.6, enough iron from the sump water was available for the algae to remove most of the available nitrogen. Complete nitrate-nitrogen removal was achieved in approximately 11 days with 3 mg/ℓ Fe addition and either 2, 4, or 8 mg/ℓ P.

The results of these experiments on the effect of CO_2 addition, flask size, etc., are preliminary and need verification. Bryte Laboratory, Department of Water Resources, is building a light box that will have both shaking and aeration facilities and will do some further experimentation along the lines of procedural evaluation. At Firebaugh we have been using the data from these batch assays to estimate concentrations of nutrients used in 1,000-gallon outdoor pools and a 1/4-acre high-rate pond; and thus far, it appears as if the light box results can be applied to larger units.

Two other items concerning technique were indirectly brought out in this study. One was the use of the specific ion electrode to measure nitrate-nitrogen. Figure 12 shows a plot of electrode reading versus brucine nitrate-nitrogen (mg/ℓ). The coefficient of correlation was 0.987, with much of the variation resulting from the brucine analyses. With this type of measurement nitrate can be rapidly and easily measured and the sample returned intact to the growth unit. One limitation of the use of the probe is its sensitivity to changes in temperature, but temperature correction curves can be prepared to eliminate this difficulty. With the high initial nitrate values in the Firebaugh water, measurement of this parameter can be a rapid and reliable estimate of algal activity. Another measurement of growth was *in vivo* fluorescence. This method differs from the usual extractable chlorphyll procedure in that the sample of live algae is read directly in the fluorometer. The advantages are ease and sensitivity at low population levels of algae.

The experimental data demonstrated that the rate-limiting factor in the light box studies at Firebaugh was probably carbon. In subsequent nutritional studies, the cultures have been artificially aerated to eliminate carbon as a limiting factor. We think that the element being studied should be the only factor limiting growth of the algae — light and other nutrients being supplied in excess. Aeration has an additional benefit in that, because of the faster growth rate, we are able to run more studies in the limited time available. Preliminary indications from adding carbon dioxide to outdoor ponds indicate that this element may be rate limiting in these units.

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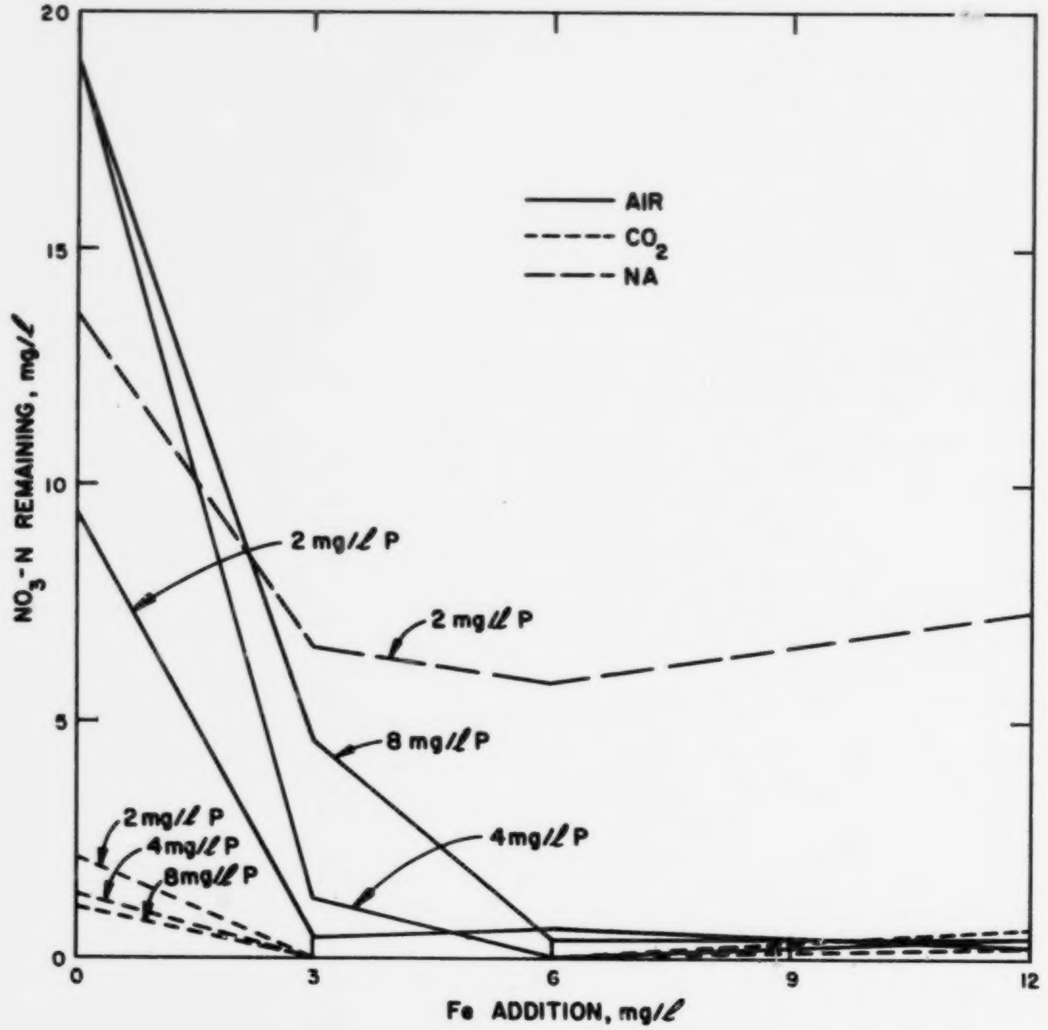

FIGURE 11. EFFECT OF IRON AND PHOSPHORUS ADDITIONS AND TYPE OF AERATION ON NITRATE UTILIZATION BY *Scenedesmus* CULTURES AT FIREBAUGH, AVERAGE OF 3 REPLICATIONS

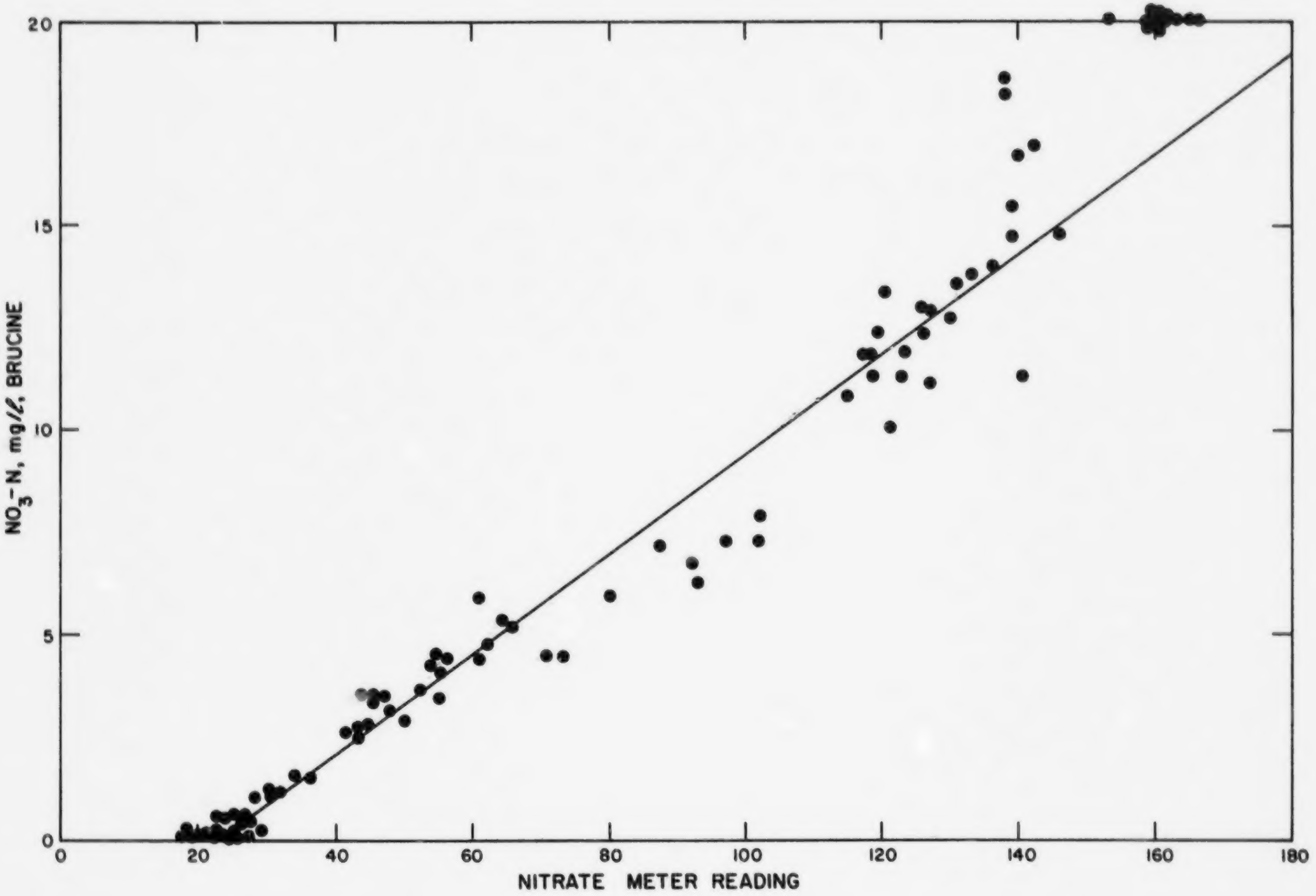

FIGURE 12. COMPARISON OF BRUCINE NITRATE VALUES WITH SPECIFIC ION ELECTRODE READINGS

ALGAL PHYSIOLOGY SESSION

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National Eutrophication Research Program
Pacific Northwest Water Laboratory
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ENVIRONMENTAL AND NUTRITIONAL REQUIREMENTS FOR ALGAE

OSMUND HOLM-HANSEN

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Before starting on my talk as entitled above, I want to take a few minutes to discuss a method for total biomass determination which should be of interest to most of us here today. The development of this biomass determination was prompted by the distribution of organic carbon in the marine environment and questions concerning the ecological significance of dissolved and detrital organic compounds in deep ocean water. The total amount of organic carbon in live cells in the oceans represents only 1% or less of the total organic carbon in the ocean, the remaining part (composing over 99%) consists of the dissolved and non-living detrital fractions. In order to estimate the microbial biomass in deep ocean water, it was necessary to develop an analytical method of great sensitivity to permit determinations on sample sizes of one to two liters. The method we have developed is based on the bioluminescent reaction occurring in firefly tails. The details of the reaction have been intensively studied by McElroy and colleagues, and have recently been summarized by Strehler (1968). For our purposes today, it is sufficient to say that the reaction is catalyzed by luciferase, and that the reactants are reduced luciferin, adenosine triphosphate (ATP), and oxygen, and the products are oxidized luciferin, adenosine monophosphate, pyrophosphate, water, and one photon of light (emission peak at about 560 mμ). As there is one photon of light emitted for each molecule of ATP which is hydrolyzed, the quantitative determination of ATP depends upon measurement of the intensity of light emission. This may be done in a variety of ways, but the system we have been using for the last four years utilizes a 14-stage photomultiplier tube operating at about 2000 volts, with the anode signal being amplified and then recorded. With this system we have a sensitivity of about 10^{-5} μg ATP, which has been sufficient for the work we have done so far in deep ocean water. At the present time we are making improvements in the apparatus and in the methodology which should permit us to lower the sensitivity to about 10^{-7} μg ATP. With this sensitivity the determination of biomass in deep ocean sediments should be possible. The four basic criteria which must be met if such a method for biomass estimation is to be useful are: 1) ATP is a constituent in all cells, whether plant or animal, 2) ATP is not associated with dead cells or with detrital material, 3) the cellular levels of ATP should be fairly uniform in all cells, and 4) a sufficiently sensitive method is available for measurement of the very small amounts of ATP which are encountered in extreme environments. On the basis of much work during the past five years, ATP apparently does satisfy these four criteria and can be very useful in estimating total biomass in any sample.

For a general discussion of the principles and apparatus involved, the reader is referred to the review by Strehler (1968), while data from natural habitats and from laboratory studies may be found in the papers by Holm-Hansen and Booth (1966), Coombs, Halicki, Holm-Hansen, and Volcani (1967), Hamilton and Holm-Hansen (1967), and Hamilton, Holm-Hansen, and Strickland (1968).

INTRODUCTION

The title which Dr. Middlebrooks gave me some months ago is so broad in scope that of necessity I have had to delete from consideration most aspects of environmental requirements of algae, and have tried to retain a few areas in which I think we all have an interest. I would like to state at the outset that many of our statements concerning algae will apply to the common photosynthetic microorganisms that we culture in our laboratorys, but will often represent over-simplifications if applied to all algae. From a nutritional point of view, algae run the entire gamut from holozoic microorganisms to those which are heterotrophic, facultative heterotrophic or autotrophic, or obligate photoautotrophic. You are all aware, I am sure, of the tremendous diversity in size and complexity of cellular organization as one proceeds from bacterial-sized unicells to the large benthic brown algae, and also of the occurrence of algae in all sorts of exotic and extreme environmental niches. I will therefore exclude from consideration all such environmental factors as water activity, light intensity and photoperiod, temperature, pressure, pH, salinity, CO_2, O_2, and N_2. I will discuss mainly the macro- and micronutrient elements and various organic growth factors which are believed to be essential for all cells, and try to relate these requirements to the questions of nutrient depletion in natural waters and to eutrophication.

The essential macronutrient elements are: carbon, hydrogen, oxygen, nitrogen, sulfur, phosphorus, potassium, magnesium, and calcium. The essential micronutrient elements are: iron, boron, zinc, copper, molybdenum, manganese, cobalt, sodium, and chlorine. Lists of essential mineral elements for plants generally do not include sodium and chlorine, but I have included them here as sodium has been shown to be essential for blue-green algae (Allen and Arnon 1955), chlorine has been shown to be a cofactor in the photosynthetic reaction (Losada, Whatley, and Arnon 1961), and nearly all marine algae require sodium chloride as a component of their nutrient solution. In addition to the above elements, silicon is known to be essential for growth of many diatoms (Lewin, Reimann, Busby, and Volcani 1966) and may perhaps be essential for all living cells. The question of vanadium essentiality remains undecided, as the claim of Arnon and Wessel (1953) that vanadium is essential for green plants has not been substantiated by other investigators. It is not known whether bromine and iodine, both of which are found as components of various organic compounds in red and brown algae, are essential for the continued growth and division of these algae, or whether these compounds represent nonessential metabolites.

Nutritional deficiencies of nearly all the essential mineral elements have been observed in terrestrial vegetation, and so I would not be surprized to find deficiencies of these elements also occurring in natural waters. In fresh waters there are many well-documented cases of mineral element deficiencies for phytoplankton growth, but in sea water it is not so clear cut. If one talks to a variety of biological oceanographers, one view which is expressed is that phytoplankton in the sea is hardly ever limited by a nutrient deficiency, and that the large variations in standing stock reflect grazing and other factors. One good argument for this view is afforded by data showing the photosynthetic rate of natural phytoplankton populations as a function of the concentration of chlorophyll. There is not much variation in the rate of photosynthesis per unit chlorophyll a regardless of the trophic characteristics of the water. The opposite point of view assumes that rapid logarithmic growth of phytoplankton is not common in ocean waters, and that a nutrient deficiency condition is common, especially in tropical and subtropical seas (see Thomas 1964, p. 289).

DETECTION OF NUTRIENT-DEFICIENT CELLS

In studies concerned with productivity in natural waters and in studies concerned with eutrophication, it is important to know the potential capacity of the water to support phytoplankton growth and to know which element(s) is limiting algal growth at any one time. There are several ways in which data of this sort may be obtained, a few of which are briefly described below.

Enrichment Experiments

In these experiments one or more nutrients are added to a sample of the water and the resulting growth is compared to the control which received no nutrient additions. A better technique, however, is to add a complete mixture minus one nutrient element to each flask, which minimizes the possibility of two or more nutrients being limiting at the same time. One must be careful, however, that no nutrients or compounds are added at inhibitory levels. In most of these experiments the water sample is used directly, but in some studies the sample is filtered and then inoculated with a sample of the natural phytoplankton population.

Using these techniques, Thomas (1966, 1969a) has obtained convincing data showing nitrogen deficiency in nutrient-poor water off Baja California. In these waters nitrate and nitrite are commonly undetectable, and ammonia occurs at a concentration between 0.1 to 0.8 µg-at/ℓ. His data also show some response occasionally to addition of phosphate, silicate, and trace metals, but lack of consistency in the data, coupled with the concentrations of phosphate and silicate in the water (1 to 4 µg-at Si/ℓ, 0. to 0.4 µg-at P/ℓ) indicate these are not clear-cut limitations as was the case with nitrogen deficiency. In upwelled water, Thomas (1969a) found no consistent effects in any enrichment experiments, inasmuch as the controls grew as well as any of the treatments.

Ryther and Guillard (1959) and Menzel and Ryther (1961) have claimed that addition of silicate and iron to Sargasso Sea water resulted in significant growth increases, while additions of nitrogen or phosphorus had little or no effects. The concentrations of these nutrients in the surface water samples were generally in the range of 0.1 to 0.5 µg-at N/ℓ, 0.1 µg-at P/ℓ, and 1.0 µg-at Si/ℓ.

Goldman *et al*. (1961, 1964, 1967), working in various fresh water lakes, have claimed demonstration of nutritional deficiencies of Mg, N, P, K, S, Mo, Fe, Zn, Mn, Co, and vitamin B_{12}. We will hear more about some of these experiments when Dr. Goldman presents his talk on *in situ* bioassay experiments.

There has been a large amount of such nutrient-enrichment experiments done in fresh water and in sea water, but only the above reports have been specifically mentioned to indicate the general type of results one encounters in the literature. I have reservations concerning the validity of many of these enrichment-type experiments, the most important points being listed below.

a. In these experiments one generally encloses the water sample in a bottle or container ranging in size from less than one liter to a 20-liter carboy. The assumption that what is found in the bottle will also be true for the lake is not necessarily valid for many reasons. If one measures the concentration of nitrate and phosphate in nutrient poor ocean water, one often finds concentrations that would support the observed phytoplankton growth rate for between 0 to a few days. If you measure the rate of photosynthesis, however, it does not decrease as one would predict from the nutrient concentrations. The rate of photosynthesis, when expressed per unit chlorophyll *a*, is often comparable to the rate found in nutrient-rich water. Thus, Eppley (1968) has reported that the rate of photosynthesis in nutrient-poor water (no detectable nitrate) off Peru was 6.5 mg C/mg chl. *a*/hr, as compared to a rate of 6.8 mg C/mg chl. *a*/hr in water samples enriched with a nutrient mixture. These results are readily explained when you consider the differences between cells confined in a bottle and those suspended in a lake or ocean. The nutrient supply to naturally-growing phytoplankton cells is continually being replenished by a variety of routes (nutrients being transported up from deeper water by diffusion or convection currents, etc., nutrients being transported up from deeper water by migration of plant or animal cells, and the continual remineralization processes occurring in the euphotic zone, which would include all grazing, parasitism, excretion of organic compounds by cells, micro-organismal degradation, autolytic processes, etc.). There is thus an equilibrium established between nutrient removal and nutrient replenishment. In the bottle test, however, the nutrients are soon depleted and the routes of replenishment have been cut off, resulting in nutrient limitation. It is obvious that the element of time is of the utmost importance in interpretation of results from such bottle tests. If the incubation period is long (a week or more), one is deviating far from reality in regard to what is happening in the lake or ocean, and if one attempts to minimize bottle effects by working with radiocarbon techniques and reducing the incubation period to a few hours, one runs into the danger of artifacts produced by the handling of the sample. When obtaining the sample, filling the bottles, and adding nutrients, the cells are being exposed to unnatural stresses, and if one measures the growth response within a few hours one might well obtain peculiar results due to cell shock, lag periods, enzyme induction periods, etc. Let me cite a few samples from the literature to illustrate a few of these points: 1) Thomas (1969b), in his enrichment experiments off Baja California, measured growth in bottles for 5 days usually, but he commonly noted a one-day lag before any growth was obtained in the bottle. In those tests, when he measured the growth increment after one day, there was generally no effect of nutrient addition. 2) Menzel and Ryther (1961), on the other hand, could detect Fe deficiency only in one-day incubation periods with Sargasso Sea water. They claimed Fe was limiting for the first day, and then the cells also became deficient in N and P during the second and third days. 3) Goldman (1964) plotted a curve showing the response of lake water to the addition of Fe during a 120-hour incubation period. At the end of ten hours, there was no significant difference between his four flasks (control, 5 ppb Fe, and 10 ppb Fe, all in the light, and 10 ppb Fe in the dark), and it was only in the period between 10 and 120 hours that growth differences were observed. What would Goldman or Thomas have found if they had cut their experiments at 12 hours, or what would Menzel and Ryther have concluded if they had measured growth after three days instead of one day? It is this sort of time-factor consideration that must be kept in mind in all bottle tests.

b. The lack of reproducibility in many experiments, coupled with some peculiar observations, shed considerable doubt as to interpretation of some of these enrichment experiments. Thus, Menzel, Hulburt, and Ryther (1963) have claimed that aluminum could replace Fe in the enrichment experiments they performed in the Sargasso Sea. There is absolutely no experimental evidence that aluminum is an essential nutrient for any plant or animal cell, and hence this effect of Al must be interpreted as indicating a chemical or charge interaction between nutrients in solution (or on membranes) which results in increased or decreased availability of certain essential elements. Some of the peculiar results of Thomas (1969a) must also be considered in this way, as he reports that the complete nutrient mixture minus Fe, Co, or EDTA results in growth up to five times that found in the control flasks (which had the entire nutrient mixture).

c. The amount of nutrient added per liter should be carefully considered by the investigator so as to avoid toxic concentrations. Elements such as Mo and Co are often added to give final concentrations of about 10 ppb, but laboratory data with algae indicate that these elements at a concentration of about 50 ppt can result in 500 mg dry weight of cells per liter. Vitamin B_{12} is often added to give 0.5 $\mu g/\ell$, which is about 1000 times the concentration generally found in sea water, and is sufficient to give you about 10 g dry weight of cells. It is necessary of course to consider the activity of the nutrient added and not just the total amount added per unit volume, as chelation, etc., might render some fraction of the nutrient unavailable for the algal cells. Estimates of such "unavailability" could, however, be easily obtained by laboratory investigations.

d. The bottle method to determine nutrient deficiencies also suffers from various indirect effects such as the influence of the ratio of monovalent to divalent ions, trace impurities in the chemicals used, chelation and percipitation phenomena, etc.

Bottle Tests With Selected Algal Strains

These tests are similar to the above enrichment experiments except that isolated algal strains are generally used to inoculate the water sample instead of using the natural population. The growth of the alga is then followed by a variety of means until maximum growth is obtained, which may take up to two weeks. The amount of algal growth is then assumed to indicate the capacity of that water sample (with or without nutrient additions) to support algal growth. This "growth potential" is then used as a guideline in eutrophication problems and in sewage treatment (Skulberg 1964, 1966). I will not discuss these techniques as Dr. Oswald is speaking on this subject, but I would like to list a few items of particular interest regarding the interpretation of data from these tests.

a. Some of the considerations I listed above for the enrichment experiments (particularly sections a, c, and d) also apply to these PAAP tests (PAAP = Provisional Algal Assay Procedure).

b. The use of a few selected algal strains (usually *Selenastrum* sp.) complicates the interpretation of the data as cells found in the natural population might have significantly different abilities to grow and assimilate organic or inorganic nutrients as compared to the laboratory test organism.

c. In the PAAP procedure, the test material is generally filtered and only the soluble fraction is added to the assay bottle. This seems to me to be a serious deficiency of the method as it ignores the interaction between dissolved and particulate fractions in natural water samples, and also completely disregards the contribution of the solid fraction when it ultimately is remineralized through microbial action.

d. The complexity of interpretation of data coming out of the PAAP tests can be appreciated by consideration of the growth increments caused by adding a set amount of sewage effluent to waters of various lakes. Thus, Skulberg (1968) and Baalsrud (1967) have described situations where 0.5 mℓ of sewage effluent added to water from a eutrophic lake causes the same growth increment brought about by the addition of 100 mℓ of the effluent to water from an oligotrophic lake. Why there should be such a large variation of up to 200-fold in growth response to added effluent is not known, though it most likely reflects the complexity of ionic interactions in nutrient solutions which affects the availability of nutrients to the algal cells.

Chemical Methods

In contrast to the above methods, the ones mentioned below depend upon an analysis of cells for some chemical constituent at the time of sampling and do not depend upon a rate measurement as do the above procedures. The assumption is that with nutrient deficiency there is generally an alteration in the chemical composition of the cell or in the activity or concentration of one or more cellular enzymes. There has not been very much work in this area relative to the efforts to show nutrient deficiencies by enrichment experiments, but the following data have been obtained in field studies.

a. We know from laboratory experiments that algae can undergo gross alterations in chemical composition as a result of nutrient deficiencies under certain light and temperature conditions (see Burlew 1953, for background information). An illustration of the effect of nitrogen deficiency on chemical composition of various marine phytoplankton under constant light and temperature conditions may be found in the article by Holm-Hansen, Sutcliffe, and Sharp (1968). The organic carbon/organic nitrogen ratio in the cells before nitrogen limitation is between 5 to 8, while the C/N ratio increases to over 20 within 10 to 14 days after onset of N deficiency. In experiments in which cells were kept for 3 to 4 weeks after depletion of nitrate from the medium, the C/N ratio has been observed to exceed a value of 40. In cells deficient in a nitrogen source either carbohydrate or lipid may accumulate within the cells, depending on the species and the environmental conditions. In diatoms deficient in silicon, lipid may continue to be synthesized, while the cellular amounts of protein and carbohydrate remain fairly constant (Coombs, Darley, Holm-Hansen, and Volcani 1967).

In examining natural populations, however, one does not generally find sufficient variation in chemical composition to permit a firm conclusion as to any definite nutrient deficiency. In all my work in the marine environment, the C/N ratio of the particulate matter in the euphotic zone is nearly always between 5 to 10, regardless of the nutrient condition of the water mass. In natural oceanic populations it is not too surprising that no pronounced alteration of the C/N ratio is found, because not only do you have a mixed population in any sample, but the amount of detrital organic material often is more abundant (on a weight basis) than the amount of living cellular material. This sort of approach has been used by Gerloff and Skoog (1954) at Wisconsin, their results indicating that in the Madison Lake, N is much more apt to be limiting the growth of planktonic blue-green algae than is P.

b. If nutrient deficiencies cause alterations in chemical composition and enzymatic composition of cells, it is possible that such deficiencies would also be reflected in alterations in morphological or cytological appearances of the cells. This assumption was made by Holmes (1966), who attempted to demonstrate nutritional deficiencies in standing stock of oceanic phytoplankton by microscopic examination. His studies were correlated with laboratory investigations of cultures grown under deficiencies of N, P, or Si. Although Holmes claimed to be able to correlate N and P deficiencies to number and arrangement of cellular organelles, he has not been successful in applying such observations to field conditions. On the basis of the inherent difficulties in this work, it does not look like a promising method.

c. In contrast to the above methods, a very promising approach, however, is to look at specific enzymes within cells or at specific components which might be correlated with the N or P status of the cell. Fitzgerald and co-workers (1969) at Wisconsin have described methods based on the cellular content of orthophosphate and alkaline phosphatase activity to indicate the nutritional status of the cell in regard to P. This approach has also been used by Eppley, Coatsworth, and Solórzano (1969) in which they attempt to use the cellular contents of nitrate reductase to indicate the nutritional status of the cells relative to various N sources.

NUTRIENT UPTAKE KINETICS BY CELLS

One very promising approach I think to the entire field of algal productivity and species succession is the study of ecologically important species in regard to their physiological adaptation to their environment. The problem is very similar to the problem of the ability of bacterial species to grow heterotrophically at the very low substrate concentrations found in deep ocean water. For this type of work batch cultures may be employed, but the use of open-system chemostats offer some advantages. It is possible by these techniques to determine the substrate concentration which permits maximum growth of any species under any given set of environmental conditions. From the kinetic data obtained, coupled with application of standard enzyme kinetics, it is easy to obtain K_s values (K_s value equals the substrate concentration which allows for one-half the maximum growth rate). These values, which will vary of course from species to species, can then be compared to concentrations found in natural conditions. The different ways of plotting such kinetic data have already been discussed at this conference by Drs. Porcella and Pearson. The work of Eppley, Thomas, and others have given us data on K_s and S_{max} for nitrate, nitrite, ammonia, and phosphate for a variety of fresh water and marine algae. The K_s values for nitrite and nitrate uptake are very interesting in that the few fresh water forms which have been investigated have higher values than do marine species; and within marine species, the values get lower as one goes from estuarine species to coastal species to open-ocean species (Eppley and Coatsworth 1968). Dugdale (1967) has recently discussed the application of such kinetic data to productivity studies and discussed a very simplified model to incorporate such data so as to be able to make predictions between nutrient concentrations, standing stock, and growth rates. Eventually, by the acquisition of more data concerning the relationships between nutrient concentrations and growth of various phytoplankton cells, coupled with data showing growth as a function of light intensity and light period, temperature, etc., it should be possible to construct mathematical models which can be used to predict productive capacity of various species for different water masses.

The sparse data we have at present concerning K_s values for N and P in the marine environment correlate nicely with some of the data from the nutrient enrichment experiments. Thus, in the nutrient-poor equatorial Pacific, one would predict N deficiency and not P deficiency, which is just what Thomas has found by his enrichment experiments (Thomas 1969b, Thomas and Dodson 1968).

Fitzgerald (1969) has recently described a method for detection of nitrogen-limitation in algae by studying the rate of absorption of ammonium-N in the dark.

ORGANIC GROWTH FACTORS

One area of great importance which I have ignored today is that of heterotrophy, or the uptake of organic solutes by plant cells. As Dr. Scher will deal with this subject later, I will not discuss it other than to say that the only organic nutrients which are known to be important for a variety of fresh water and marine algae are vitamins B_1 (thiamine), B_{12} (cobalamine), and biotin. There are very sensitive bioassay procedures known for these three vitamins (Carlucci and Silbernagel 1967) which permit the determination of them in any samples of water. Judging from the quantitative distribution of these vitamins in sea water and fresh water (Carlucci 1969; Daisley 1969) coupled with their contents in cellular material, it appears unlikely that a nutrient limitation of these vitamins is often encountered in the marine environment. It is not known, however, how the chemical composition of the water may affect the availability of the vitamin for assimilation by algal cells. Various aspects of vitamins in regard to productivity in coastal waters have recently been discussed by Carlucci (1969).

In closing, I would merely like to add that our knowledge in regard to productivity as related to nutrient supply is rather meager. Let me cite one example which impressed me considerably. Last September Robert Barsdate and I were at Pt. Barrow (Alaska) at a time when there was a very extensive red tide which was caused by a pigmented ciliate about 150 microns in length. The distribution of phytoplankton and microzooplankton in or out of the red tide patches was quite similar except for this one species of ciliate which reached concentrations as high as 600 individuals per mℓ in the red water. This ciliate, which apparently is a photoautotrophic cell by virtue of endosymbiotic algae of unknown taxonomy, has not been reported previously, and has not been seen in collections made during the past six months. We are completely unable to explain such a phenomenon at the present time, but I intuitively feel that the answer to some of these problems will lie in the area of vitamins or other organic growth factors and not in the area of inorganic nutrition.

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NITROGEN FIXATION ASSESSMENT BY ACETYLENE REDUCTION

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Biological nitrogen fixation is the enzymic conversion of molecular nitrogen to ammonia. Until recently, biological nitrogen fixation was measured by the following: 1) increase in total N when the organism was grown in the absence of or on limited fixed nitrogen, 2) incorporation of ^{15}N-enriched N_2 into an organism, 3) incorporation of ^{13}N-enriched N_2 into an organism, or 4) ammonia formation from N_2. Of these four methods, only the first three are applicable to the measurement of nitrogen fixation in intact systems. The latter method has been used with cell-free systems but it cannot be used with intact organisms because of the rapid incorporation of the synthesized ammonia into amino acids.

Growth on N-free medium has been used for screening for N_2-fixing agents but this method lacks sensitivity and requires extended periods of investigation. Although considering the inadequacies of this method, it is the most valid method for studying the importance of fixation in an ecosystem. The use of ^{15}N for the measurement of nitrogen fixation was introduced in 1941 by Burris and Miller (1941). Using the limits described by Burris and Wilson (1957), the ^{15}N method is at least 60 times more sensitive than measuring the increase in total nitrogen. The method is an absolute measurement of nitrogen fixation and has been used extensively for measuring *in situ* fixation; however, it is tedious and expensive. The ^{13}N technique (Nicholas *et al.* 1961; Campbell *et al.* 1967) depends upon the measurement of a radioactive product and consequently is extremely sensitive. Nevertheless, because of the short half-life of ^{13}N (10 minutes), the method is of little value in ascertaining *in situ* fixation.

The nonspecificity of the active site of the nitrogen-fixing complex was recognized by Wilson and Umbreit (1937) when they demonstrated H_2 specifically inhibited nitrogen fixation. Mozen and Burris (1954) demonstrated, by use of $^{15}N_2O$, an incorporation of ^{15}N into *Azotobacter vinelandii* and soybean root nodules, and their observations were verified by Hoch *et al.* (1960). Because these studies were done with intact cells and nodules, the true significance of the finding was obscured. With cell-free extracts of *A. vinelandii* and *Clostridium pasteurianum,* Schöllhorn and Burris (1966) observed that azide was reduced to NH_3, and that acetylene was reduced when the extracts were furnished with a reductant and ATP. Dilworth (1966) independently demonstrated acetylene reduction by cell-free extracts of *C. pasteurianum*, and he established that the product of acetylene reduction was ethylene.

The discovery by Schöllhorn and Burris (1966) and Dilworth (1966) that the nitrogen-fixing complex reduces acetylene to ethylene is the basis for a new and sensitive assay for nitrogen fixation. The general procedure is to expose the organism to be tested to acetylene, incubate, and analyze the gas phase for ethylene. Although a number of methods can be used to measure ethylene, one of the most sensitive methods is by H_2-flame ionization gas chromatography as suggested by Hardy and Knight (1967).

Several lines of evidence strongly suggest that acetylene, which is isoelectronic and isosteric with N_2, is reduced by the nitrogenase:

1. Identical requirements for nitrogen fixation and acetylene reduction with intact organisms (Koch and Evans 1966; Stewart et al. 1967, 1968; Hardy et al. 1968).

2. Identical requirements for nitrogen fixation and acetylene reduction with cell-free extracts: ATP, reductant, and extracts of N_2-grown cells (Schöllhorn and Burris 1966; Dilworth 1966; Koch et al. 1967).

3. Similar inhibition of ATP-dependent H_2 evolution by N_2 and acetylene (Burns and Bulen 1965; Hardy et al. 1968).

4. Competitive inhibition of acetylene reduction and nitrogen fixation by CO (Hardy et al. 1968; for contradictory results see Hwang and Burris 1968).

5. Competitive inhibition of N_2 fixation by acetylene (Schöllhorn and Burris 1966, 1967; for contradictory results see Hwang and Burris 1968).

6. Similar activation energies (13 to 15 k-cal/mole above 20° and 35 to 50 k-cal/mole below 20°) for both reductions (Hardy et al. 1968).

7. Requirement of both molybdoferredoxin (fraction 1) and azoferredoxin (fraction 2) of the nitrogen-fixing complex from Azotobacter vinelandii (Kelly et al. 1967a), Clostridium pasteurianum (Moustafa and Mortenson 1968; for contradictory results see Taylor 1969) or bacteroids from soybean root nodules (Klucas et al. 1968) for the reduction of nitrogen or acetylene.

Because of its recent development, the acetylene reduction assay has not been utilized extensively. However, a variety of biological systems listed in Table I have been shown to reduce acetylene to ethylene. With the exception of Aphanizomenon flos-aquae, all of these systems have been shown previously to fix N_2. Stewart, Fitzgerald, and Burris (1967, 1968) used the acetylene reduction assay to perform in situ studies with common lake algae, root nodules, soil samples, and pure cultures of blue-green algae. They correlated daily and hourly variations of acetylene reduction activity with the abundance of the nitrogen-fixing alga, Gloeotrichia echinulata, and studied the effects of light and dark incubations of algae samples. Hardy et al. (1968) performed an extensive study to evaluate the acetylene reduction assay under laboratory and field conditions using purified nitrogenase preparations and pure cultures of N_2-fixing bacteria. They concluded that the acetylene reduction assay was a valid measurement for nitrogen fixation. Schwinghamer, Evans, and Dawson (1969) used the assay for evaluating the effectiveness in mutant strains of Rhizobium relative to other criteria of N_2 fixation. The acetylene reduction technique also has proven useful in measuring nitrogenase activity during purification of nitrogenase from Azotobacter vinelandii (Kelly et al. 1967a) and bacteroids from soybean nodules (Koch et al. 1967; Klucas et al. 1968).

Russell (1969) found that several lichens which are known to fix nitrogen also possess the capability to reduce acetylene to ethylene.

TABLE I

NITROGEN-FIXING ORGANISMS EXHIBITING ACETYLENE REDUCTION TO ETHYLENE

Free-Living Microorganisms

Bacteria
- Clostridium pasteurianum (Dilworth 1966)
- Azotobacter vinelandii (Schöllhorn and Burris 1966)
- Azotobacter Chroococcum (Kelly et al. 1967b)
- Rhodospirillum rubrum (Munson and Burris 1969)

Blue-green algae
- Anabaena cylindrica (Stewart et al. 1968)
- Anabaena flos-aquae (Stewart et al. 1968)
- Aphanizomenon flos-aquae (Stewart et al. 1968)
- Calothrix sp. (Stewart et al. 1968)
- Gloeotrichia echinulata (Stewart et al. 1968)
- Nostoc entophytum (Stewart et al. 1968)
- Nostoc muscorum (Stewart et al. 1968)
- Mastigocladus laminosus (Stewart et al. 1968)
- Tolypothrix tenuis (Stewart et al. 1968)

Symbiotic Systems

Root nodules and root-associated microorganisms
- Glycine max (Koch et al. 1967)
- Myrica cerifera (Sloger and Silver 1967)
- Phaseolus vulgaris (Hardy et al. 1968)
- Medicago sativa (Hardy et al. 1968)
- Arachis hypogea (Hardy et al. 1968)
- Pisum sativum (Hardy et al. 1968)
- Comptonia peregrina (Stewart et al. 1968)
- Alnus rugosa (Stewart et al. 1968)
- Cycas revoluta (Stewart et al. 1968)
- Zamia floridana (Stewart et al. 1968)

Lichens
- Collema sp. (Russell 1969)
- Peltigera sp. (Russell 1969)
- Pseudocyhellaria sp. (Russell 1969)

Hardy et al. (1968) recently listed the advantages of the acetylene technique compared to the other methods for determining nitrogen fixation. These advantages can be summarized as the following:

1. Sensitivity — Under optimal conditions, the minimum detection limit of ethylene is less than 10^{-12} mole per sample; the acetylene technique therefore is at least 10^3 times more sensitive than the ^{15}N method but has about the same sensitivity as the ^{13}N method. Also, if the electron flow is the limiting step in nitrogen fixation, the same amount of nitrogenase would produce 3 molecules of ethylene for every two molecules of ammonia.

2. Cost — The ^{15}N method requires $^{15}N_2$, a mass spectrometer, and ancillary equipment while the only major pieces of equipment required for the acetylene reduction method are a gas chromatograph and a recorder. The expenditure in terms of time consumed per assay by the acetylene method is approximately 10-fold less than the ^{15}N method.

3. Mobility — Because of the nature of the assay and limited equipment requirement, *in situ* studies can be performed either by constructing a complete mobile laboratory or exposing samples in the field and returning them to the laboratory for analysis. The technique greatly simplifies field and survey studies.

4. Internal standard — Since a known amount of acetylene is used in the assay, a natural internal standard is provided for quantitation of the data.

5. Convenience — The storage stability, simplicity, and sample conservatism make the overall study of nitrogen fixation much easier with the acetylene method.

The assay does have certain inherent problems and disadvantages. One difficulty is assigning an absolute ratio for acetylene reduced to nitrogen fixed. In terms of electron consumption, the acetylene to ethylene reduction requires two electrons and the reduction of N_2 to two molecules of NH_3 requires 6 electrons. If one considers the flow of electrons as the limiting step in the reduction, a molar ratio of 1.5 would be expected for the ethylene formed to ammonia formed. In studies where acetylene reduction and nitrogen fixation were measured using cell-free extracts under comparable assay conditions, ratios of 1.25 (Schöllhorn and Burris 1967), 1.59 (Klucas 1967), 1.8 (Hardy *et al.* 1968), 2.1 (Hardy *et al.* 1968), and 1.49 (Klucas *et al.* 1968) were obtained. Table II summarizes the results of an experiment comparing C_2H_2 reduction to N_2 reduction using cell-free extracts of *A. vinelandii*, *C. pasteurianum*, and bacteroids from soybean nodules. The values range from 1.5 for the bacteroid extract to 1.9 for the *C. pasteurianum* extract. Using the ^{15}N technique for comparison of nitrogen fixation with acetylene reduction by various whole blue-green algae, Stewart *et al.* (1968) found values of 1.8 for *Nostoc muscorum*, 1.4 for *Anabaena cylindrica*, and 1.6 for *Anabaena flos-aquae*. Although most ratios center around the theoretical value of 1.5, a final evaluation of the quantitative relationship of acetylene reduced to ammonia formed requires further experimentation. A potential interference is the possible existence of enzymes other than nitrogenase that have the capability of reducing acetylene to ethylene. Although this problem has not materialized from the available data, periodic crosschecks with the ^{15}N technique should be performed. It is evident that the acetylene reduction assay measures only the immediate capability to fix nitrogen and no information is given on the potential capability of the system. Another minor disadvantage of the acetylene reduction assay is the explosive nature of acetylene but with proper handling this need not be a problem.

TABLE II

COMPARISON OF N_2 FIXATION TO ACETYLENE REDUCTION BY CELL-FREE EXTRACTS

Cell-Free Extract	Protein mg	NH_3 Formed nmoles	C_2H_4 Formed nmoles	C_2H_4/NH_3
Clostridium pasteurianum	4.2	1130	2120	1.9
Azotobacter vinelandii	11.5	1980	3330	1.7
Bacteroids from soybean root nodules	9.2	950	1430	1.5

NOTE: The C. pasteurianum, A. vinelandii, and bacteroid cell-free extracts were prepared by the methods of Carnahan et al. (1960), Bulen et al. (1965), and Klucas et al. (1968), respectively. The nitrogen fixation assay was done by the method of Bulen et al. (1965), and the acetylene reduction assay was performed as described by Klucas et al. (1968).

A method for the acetylene reduction assay, first described by Stewart et al. (1967), is used in several laboratories and is described below. The equipment required for in situ studies include: a) a gas chromatograph equipped with a H_2-flame detector and a recorder, b) reaction bottles fitted with serum stoppers, c) needles and syringes, d) cylinders of gas (high purity acetylene; a gas mixture of oxygen, argon, and carbon dioxide for aerobic studies; or argon for anaerobic studies), e) gas regulators, and f) equipment for measuring sample size and/or weight. Samples are placed into bottles and the bottles sealed with serum stoppers. Using one needle as a gas vent and another as the gas inlet, the bottles are flushed free of air with the appropriate gas minus the acetylene. After removal of the needles, the reaction is commenced by the injection of acetylene to give about 0.1 atm. of acetylene. After incubation the reactions are terminated by injection of 50% trichloroacetic acid. Gas samples are then removed with a syringe and assayed for ethylene by gas chromatography. Quantitation is accomplished by comparing peak area or peak height traced by the recorder with the area or height of known amounts of ethylene. From this information, the total amount of ethylene in the reaction bottles can be calculated from the total volume of the bottle, the temperature, and the pressure. The data can also be quantitated by using acetylene as the internal standard (assuming the total loss of acetylene to ethylene is minimal during incubation), and by comparing the peak area or height of acetylene to that found for the ethylene taking into account the difference in detectability of the acetylene and ethylene.

Three types of gas chromatographic columns have been used for the separation of acetylene from ethylene. Koch *et al.* (1967) and Hardy *et al.* (1968) used activated alumina columns at 130°. Hardy *et al.* (1968) also used a 1/8 inch x 10 foot column containing 20% ethyl, N', N'-dimethyl oxalamide on 100 to 120-mesh acid-washed firebrick at 0°. Kelly *et al.* (1967a), Stewart *et al.* (1967, 1968), and Klucas *et al.* (1968) used 80 to 100-mesh Porapak R columns. A 1/8 inch x 6 foot column at 50° with a carrier gas flow rate of 25 mℓ/min will resolve acetylene and ethylene into two peaks with a total recovery time of 2 to 3 minutes.

In summary, the acetylene reduction assay for nitrogen fixation has been used successfully with a number of free-living and symbiotic systems. Because of the simplicity, sensitivity, and rapidity of the method, it is particularly adaptable to field investigations where numerous samples must be analyzed. One aspect of eutrophication is the nitrogen input into the aquatic environment. Although many sources contribute to the increase in nitrogen, *in situ* fixation by microorganisms certainly is involved in this input. With the acetylene reduction assay, a simple and sensitive method is available for ascertaining the amount of nitrogen fixation in these aquatic ecosystems.

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PHYSIOLOGICAL AND REGULATORY ASPECTS OF HETEROTROPHY IN ALGAL FLAGELLATES: CONDITIONALLY EXPRESSED CHARACTERISTICS

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INTRODUCTION

The presence of a mechanism for converting light into chemical energy in photosynthetic microorganisms has led to the unwarranted view that algae are largely obligate photoautotrophs, and that they are relatively unresponsive to exogenous low molecular weight carbon compounds. Although many algae do indeed depend upon photosynthetic CO_2 assimilation as their primary source of carbon, there is growing experimental support for the view that soluble organic compounds in the environment may serve to stimulate growth, regulate metabolism, or inhibit specific cellular functions. In this paper, the relevant literature concerned with physiological and regulatory responses of algal flagellates to organic carbon and nitrogen sources will be reviewed. In addition, recent evidence for conditionally expressed heterotrophic characteristics in *Euglena gracilis* will be presented. The review is not intended to be a compendium of all previous studies on heterotrophy in algae. For more comprehensive reviews of this subject, see Pringsheim (1959); Provasoli (1961); Danforth (1962, 1967, 1968); Cook (1968); and Barras and Stone (1968).

UTILIZATION OF EXTERNAL ORGANIC COMPOUNDS BY ALGAL FLAGELLATES

Organic Carbon Sources

Wide differences exist in the ability of photosynthetic microorganisms to utilize exogenous carbon compounds. Utilization of carbon sources other than CO_2 is under the control of genetic determinants within the cell, as well as chemical and physical factors in the cell environment. Some strains of *Euglena* (Lwoff 1951), *Chlamydomonas* (Lewin 1950), and dinoflagellates (Barker 1935) are strict photoautotrophs. Others assimilate substrates only under restrictive conditions, e.g., during growth in light (Pringsheim and Wiessner 1960; Cook 1966). Cramer and Myers (1952), Lewin (1954), and Pringsheim (1955b) have noted that some strains of *Euglena* and *Chlamydomonas* grow equally well autotrophically in the light or heterotrophically in darkness. In chrysomonad flagellates such as *Ochromonas,* heterotrophy may be either facultative or obligate (Hutner *et al.* 1953, 1957; Pringsheim 155a; Myers and Graham 1956; Aaronson and Baker 1959). Several cases of anaerobic heterotrophy have been reported. *Ochromonas malhamensis* may exhibit an anaerobic metabolism which is analogous to bacterial photosynthesis in that organic hydrogen donors are required for photoreduction of CO_2 (Vishniac and Reazin 1957). Organic carbon sources may also function as hydrogen donors for the assimilation of inorganic nitrogen compounds. Huzisige and Satoh (1960) reported that the addition of malate or pyruvate enhanced the rate of nitrite reduction in *Euglena*. Under anaerobic conditions, in the dark, *O. malhamensis* can ferment glucose to ethanol (Reazin 1956). Wiessner (1965, 1966) has reported that light-dependent uptake of acetate by *Chlamydobotrys* could proceed under anaerobic conditions in the presence of 3 - (3,4-dichlorophenyl) - 1,1-dimethylurea, and inhibitor of oxygen evolution.

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The range of compounds that support heterotrophic growth in algal flagellates includes sugars, organic acids, alcohols, amino acids, and other organic nitrogen sources. Within the euglenids, sugar utilization is dependent upon the physiological strain, as well as environmental factors. Cramer and Myers (1952) and Hurlbert and Rittenberg (1962) have demonstrated that glucose utilization occurs under conditions of high CO_2 tension. These authors, as well as Cook and Heinrich (1965), have noted that glucose is used at pH 4.5, but not at neutrality. Cells grown in the presence of most tricarboxylic acids (TCA) or closely related amino acids readily utilize sugar (Hutner *et al.* 1956; Scher and Frank 1956; Baker *et al.* 1955; Scher and Aaronson 1958). Glycolic acid fails to support growth of *Euglena* when supplied as the sole carbon source (Cramer and Myers 1952; Cook 1968). Frank and Scher (1969) have undertaken a reanalysis of heterotrophy in several strains of *E. gracilis* in order to more clearly define the prerequisites for substrate utilization. All TCA cycle intermediates but citrate and cis-aconite supported growth of the *z* strain when supplied as the sole carbon source at pH 3.4 to 3.6. These compounds also stimulated glucose utilization. In Table I the growth of the *z* and *Visher* or *Mainx* strains are compared in media containing lactate, glycolate, or succinate as sole substrates, and in combination with glucose. With strain *z*, some growth was observed with glucose in darkness; none occurred in the light. When tested alone, both lactate and succinate permitted growth in the light as well as in darkness, and growth was enhanced in the presence of glucose. Glycolate was unable to support growth as sole substrate; however, this compound appears to be an effective inducer of glucose utilization. By contrast, none of these substrates supported growth of the *Visher* strain in darkness. Furthermore, growth in light was independent of the organic carbon source supplied. The results of experiments with the *Visher* strain clearly indicate a light-dependent obligately autotrophic metabolism. Schou *et al.* (1950) have interpreted glycolic acid-2-^{14}C tracer studies in *Scenedesmus* as evidence that this compound enters the TCA cycle. App and Jagendorf (1963) and Cook and Heinrich (1965) have presented evidence that glucose utilization is adaptive. Belsky (1957) has also reported that hexokinase is "labile" in the absence of glucose. These results taken together indicate that one (or more) enzymes involved in the metabolism of glucose are inducible. They also suggest that CO_2, tricarboxylic acids, or compounds that readily enter the TCA cycle to release CO_2, may function as inducers of glucose metabolizing enzymes, whereas induction by glycolate may be gratuitous.

Organic Nitrogen Sources

Amino acids that can serve as sole nitrogen sources for *E. gracilis* have been surveyed by Buetow (1966). He notes that neutral aliphatic amino acids and dicarboxylic amino acids and their amides were most effective at pH 6.8 with

TABLE I

EFFECT OF SUBSTRATES ON GLUCOSE UTILIZATION IN PHYSIOLOGICALLY DIFFERENT STRAINS OF *Euglena gracilis*

LIGHT

	Visher or *Mainx* Strain		*z* Strain	
Substrates 0.4 mg/mℓ	Control	Glucose 1.0%	Control	Glucose 1.0%
None	+	+	0	0
Lactate	+	+	++++	++++
Glycolate	+	+	0	++++
Succinate	+	+	++	++++

DARKNESS

None	0	0	0	++
Lactate	0	0	+++	++++
Glycolate	0	0	0	++++
Succinate	0	0	++	++++

Legend:
0 = Optical Density (OD) < 0.2
+ = OD 0.2-0.7
++ = OD 0.8-1.3
+++ = OD 1.4-1.9
++++ = OD > 2.0

Experimental Conditions: Cells of *E. gracilis* were grown in medium of Hutner *et al.* (1956) modified as follows: All organic compounds were omitted except for thiamine and vitamin B_{12}. Nitrilotriacetic acid 0.08 mg/mℓ was added. The methods used for microbiological assay of substrates have been described in detail elsewhere (Hutner *et al.* 1958a). After 6-8 days at 25-28°C growth was measured as optical density with a Welch densichron equipped with a cuvette holder and a red-sensitive phototube (Baker *et al.* 1955).

acetate as the major carbon source. In addition to amino acids, other organic nitrogen compounds may be utilized by algal flagellates. Urea can serve as sole nitrogen source for Chlamydomonas reinhardi (Sager and Granick 1953). Evidence for adenine and guanine incorporation into Euglena has been reported (Brawerman and Chargaff 1959; Bolton et al. 1963; Mego 1964a; Sagan 1965; Sagan et al. 1965; Dubash and Rege 1967). Reversal of 5-fluorouracil inhibition by uracil has been demonstrated (Evans and Smillie 1962; Smillie 1963; Smillie et al. 1963), and inhibition of uracil incorporation by actinomycin D has also been observed in Euglena (Pogo and Pogo 1964; McCalla and Allen 1964). Two analogs of thymine have been reported to influence chloroplast replication in Euglena. Cells grown in the presence of azathymine show reduced sensitivity to loss of chloroplasts induced by sublethal doses of ultraviolet light (Lyman and Smillie 1963). Chloroplast loss in cells grown in the presence of sublethal concentrations of sulfanilamide is markedly increased upon addition of 5-bromouracil, an analog of thymine (Scher and Collinge 1965).

Other Functions of Exogenous Organic Compounds

In addition to serving as auxiliary sources of carbon and nitrogen, Provasoli (1958) has reported that organic compounds can serve as phosphorus sources for algae. Hutner et al. (1966) have employed glycerophosphate as sole phosphate source for Euglena in neutral to alkaline media. Glycerophosphate can serve as both pH buffer and a source of phosphate, to eliminate precipitation of inorganic phosphate.

As noted above, organic compounds may function as pH buffers. Hutner et al. (1950, 1966), Provasoli (1961), and Wetzel (1966) have also called attention to the role of organic compounds as chelating agents for solubilizing divalent cations.

REGULATORY ASPECTS OF HETEROTROPHY IN ALGAL FLAGELLATES

In the presence of utilizable organic carbon or nitrogen sources, there are two mechanisms that may function to control enzymes that are not contributing to the growth of the cell: 1) The synthesis of inducible enzymes may be repressed by the product or products of the enzyme-catalyzed reaction (repression); and/or 2) the activity of preexisting enzymes may be inhibited by the end product(s) of the biosynthetic pathway (feedback or end-product inhibition).

Repression of Inducible Enzymes

In photosynthetic microorganisms, both dark and light metabolism may be subject to repression. There is now abundant evidence that chloroplast development is inducible in Euglena (Schiff and Epstein 1965), Ochromonas (Gibbs 1962), and mutants of Chlamydomonas (Weaver 1952). The formation of photosynthetic pigments and enzymes is induced by light and repressed by darkness or the presence of utilizable organic compounds. Chlorophyll formation in Euglena is inhibited by ethanol, acetate, or malic acid (App and Jagendorf 1963). When cells of Chlamydomonas mundana are grown in the presence of acetate, enzymes of the carbon fixation cycle are repressed (Russell and Gibbs 1966). In short term experiments with E. gracilis, the amount of $^{14}CO_2$ fixed in cells grown in the presence of lactate in light is strongly reduced when compared with controls grown photoautotrophically with CO_2 as sole carbon source (Table II). These results support the view that enzymes required for photosynthetic CO_2 assimilation are repressed by organic substrates.

TABLE II

REPRESSION OF $^{14}CO_2$ UPTAKE IN
LACTATE GROWN CELLS OF
E. gracilis

Incorporation Time (sec)	CO_2 Grown Cells	Lactate-Grown Cells
15	4.89	1.74
	4.68	1.75
60	21.53	12.52
	21.24	12.64

cpm x 10^3/50λ sample

Experimental Conditions: Cells of *E. gracilis z* were grown in the light at 26-28°C in the medium of Hutner *et al.* (1956) modified as follows: All organic compounds were omitted, and disodium ethylenediaminetetraacetate 0.5 g/ℓ was added. The medium was diluted to 0.5 strength and DL-lactic acid 1 g/ℓ was added for lactate-grown cells. Log phase cells were centrifuged at 3000 rpm, washed, and resuspended in distilled water in a 55-mℓ volume flattened separatory funnel at a final concentration of 2 mℓ packed cell volume/50 mℓ. The cell suspension was flushed with 5.0% CO_2 in air for 10 minutes, then with air for 1 minute. 50 μℓ of $NaH^{14}CO_3$ (50 μc) was added and the cell suspension was vigorously agitated between two 150 W. spotlights shielded by circulating water filters. Samples were extracted in 80% ethanol with a few drops of acetic acid added to drive off excess CO_2. The ethanol extracts were steam distilled to reduce the volume of residue to 5.0 mℓ, and the radioactivity in 50λ of each sample was counted.

Evidence for End-Product Inhibition of Amino Acid Biosynthetic Enzymes in *Euglena*

Studies on the control of biosynthetic enzymes by end-product inhibition have been reported in bacteria (Umbarger 1956), fungi (Wagner *et al.* 1958), and a few higher plants (Davies 1964; Oaks 1965). However, little information is available for algae. The observation by Aaronson and Bensky (1962) that O-methylthreonine, an analog of isoleucine, selectively inhibits chloroplasts in *Euglena*, prompted us to investigate the enzymes concerned with isoleucine biosynthesis as possible sites of action of the analog. Kempner and Miller (1965) have followed the fate of uniformly labeled ^{14}C – amino acids into protein of E. gracilis. They reported that ^{14}C-labeled threonine was converted to isoleucine, leucine, and glycine. Using cell extracts of E. gracilis, we have examined the enzymatic conversion of threonine into isoleucine. When threonine is added to cell extracts, α-ketobutyric acid is the first product that can be detected (Scher and Haley 1968). The formation of α-ketobutyric acid from threonine is mediated by threonine deaminase. In bacteria and yeast, this enzyme has been characterized as an allosteric protein. The enzyme contains two types of binding sites that interact with small molecules. A catalytic site binds to threonine and functions in the deamination of this amino acid to the corresponding α-keto acid. The regulatory, or allosteric, site binds to the end product isoleucine. In the presence of isoleucine, the enzyme is unable to convert threonine to α-ketobutyrate. When isoleucine is added to cell extracts of E. gracilis, the rate of α-ketobutyrate formation is markedly reduced (Scher and Haley 1968). These results are consistent with the hypothesis that Euglena threonine deaminase is sensitive to feedback inhibition by the end product isoleucine. Thus, wasteful synthesis of isoleucine is prevented because excess isoleucine inhibits the catalytic activity of threonine deaminase, the first enzyme in the biosynthetic pathway (Figure 1).

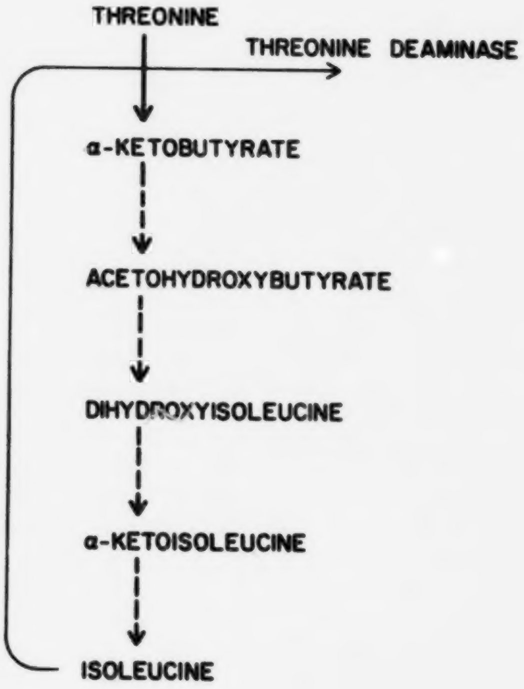

FIGURE I. THE PATHWAY OF ISOLEUCINE BIOSYNTHESIS

LATENT (OR CONDITIONALLY-EXPRESSED) AUXOTROPHIC CHARACTERISTICS OF EUCARYOTIC ORGANELLES

Recent evidence supports the hypothesis that chloroplasts (and probably mitochondria) of eucaryotic cells are resident auxotrophs (Zeldin and Schiff 1967) dependent upon products of cytoplasmic enzymes, coded by nuclear genes. However, there has been little effort directed at identifying specific auxotrophic requirements of organelles.

If the supply of metabolites for organelle biosynthesis is adequate, then no selective advantage would be conferred upon plastids or mitochondria that can code for enzymes of amino acid, vitamin, purine, or pyrimidine biosynthesis. To account for the inhibition of chloroplasts by the isoleucine analog, O-methylthreonine, we postulated that these organelles are deficient in enzymes required for biosynthesis of isoleucine. By this hypothesis, little or no threonine deaminase activity should be found in chloroplasts. To test this idea, chloroplasts were isolated from E. gracilis by sucrose density gradient centrifugation, and chloroplast extracts were assayed for their ability to convert threonine to α-ketobutyrate. Less than 1% of total cell enzyme activity was detected in the chloroplast fraction (Table III).

TABLE III

THREONINE DEAMINASE ACTIVITY IN ISOLATED SUBCELLULAR FRACTIONS OF E. gracilis z

Fraction	OD 540 min^{-1} cell^{-1}
Cells with Chloroplasts	3.0×10^{-8}
Cell Fraction: (Less Chloroplasts)	6.0×10^{-8}
Purified Chloroplasts	1.4×10^{-10}

Experimental Conditions: Cell of E. gracilis were grown in the light at 25 ± 1°C in the medium of Hutner et al. (1956), chloroplasts were isolated from ~ 20 mℓ (packed cell volume) of log phase cells by the sucrose flotation method of Eisenstadt and Brawerman (1964), and extracts were prepared in a French pressure cell. α-ketobutyrate was assayed by the method of Friedmann and Haugen (1943) at 540 mμ.

These results support the hypothesis that chloroplasts are deficient in threonine deaminase, and hence, they are unable to synthesize isoleucine from threonine. This interpretation is in agreement with the view that chloroplasts are not informationally autonomous organelles; rather they are strongly dependent upon products of enzymes directed by nuclear genes for a supply of metabolites. The latent auxotrophic character of chloroplasts cannot be detected within the cell; however, it emerges when the intracellular distribution of threonine deaminase is examined in isolated cell fractions.

A second approach to the identification of latent auxotrophy in cell organelles is suggested by studies with metabolic analogs such as O-methylthreonine. When cells of E. gracilis z are grown in analog-free media, the addition of isoleucine has no detectable effect on chloroplast replication. Under normal conditions, the frequency of spontaneous mutants lacking chloroplasts in this strain is of the order of 10^{-2} to 10^{-3}. However, when cells are exposed to O-methylthreonine, the frequency of chloroplast-less mutants in the population markedly increases. The kinetics of loss of chloroplast-forming ability in cells exposed to O-methylthreonine has been examined under isoleucine-limiting conditions. The results are shown in Figure 2. In the presence of the analog, isoleucine was found to be required for normal chloroplast replication. Under these conditions, this latent auxotrophy is expressed as a requirement for organelle continuity. We propose that the requirement for a metabolite needed to maintain organelle function in the presence of a specific analog be designated a conditionally-expressed characteristic of DNA-containing organelles that replicate semiautonomously in eucaryotic cells.

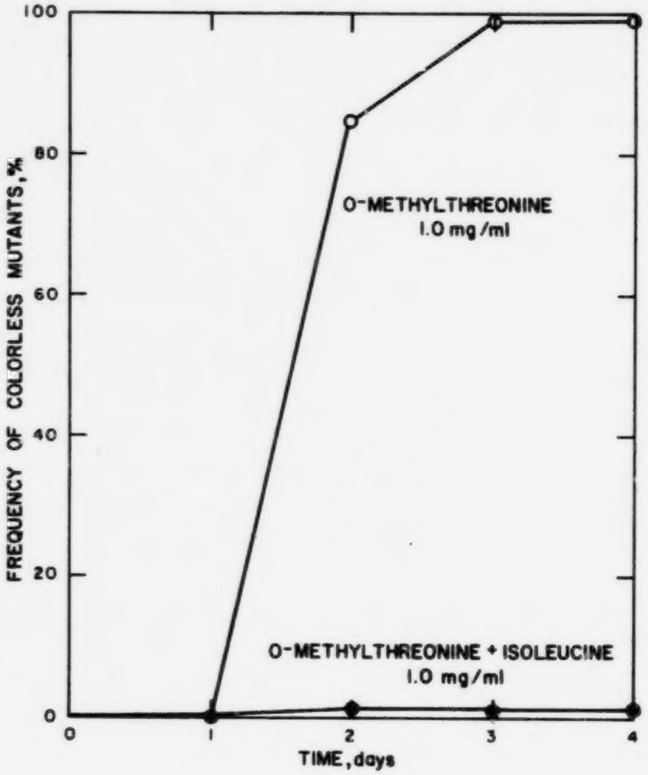

FIGURE 2. EFFECT OF O-METHYLTHREONINE AND ISOLEUCINE ON THE FREQUENCY OF COLORLESS MUTANTS

Experimental Conditions: Log-phase cells were incubated with the analog and/or the metabolite, samples were withdrawn at intervals during the incubation period, diluted to ~ 200 cells/0.1 mℓ, and plated as described previously (Lyman et al. 1961; Scher 1966b).

Schiff and Epstein (1965) have postulated that the replication of organelles, such as the chloroplast, is responsive to signals which are necessary for the coordination of nuclear, organelle, and cell division. The identification of a latent or conditionally-expressed isoleucine requirement for chloroplast replication in Euglena strongly suggests that the rate of amino acid biosynthesis may provide a mechanism through which nuclear genes exert control over organelle replication.

Similar observations of analog-induced modification of organelle replication have been reported in yeast as well as algal flagellates. In the presence of 5-fluorouracil, a high frequency of cytoplasmic petite mutants is detected in yeast (Moustacchi and Markovitch 1963). When uridine is added, the mutagenic effect of this analog is depressed. In certain petite mutants of yeast, the mitochondrial DNA is reported to be altered (Tewari et al. 1966). Dubash and Rege (1967) reported an increase in chloroplast-less mutants when Euglena was grown in the presence of 5-fluorouracil.

We have also noted (Scher and Collinge 1965) that chloroplast-less mutants are generated with high frequencies when Euglena are grown in the presence of sulfanilamide (Figure 3). The mutagenic effect of sulfanilamide is markedly reduced by p-aminobenzoic acid (Figure 4). These results provide additional support for the hypothesis that organelles are resident auxotrophs and that latent auxotrophic characteristics can be detected when specific deficiencies are imposed by analogs of vitamins or growth factors.

Supraoptimal Temperature As An Experimental Tool For The Analysis Of Conditionally Expressed Characteristics

The conditional approach is based on the idea that genetic defects are expressed under one set of conditions (restrictive), but not another (permissive). This approach has been successfully employed to identify latent temperature-sensitive elements in algal flagellates.

When cells of Euglena or Ochromonas are grown at restrictive temperatures (in this case, above their normal optimum for growth) two classes of temperature-sensitive defects can be recognized:

1. Dispensable, auxotrophic characteristics that can be by-passed nutritionally by providing the immediate product or end product of the blocked reaction.

2. Indispensable, nonauxotrophic characteristics that cannot be overcome by addition of low molecular weight compounds to the medium.

Temperature Induced Auxotrophy

Two distinct types of auxotrophic requirements can be distinguished when cells of Euglena are grown at temperatures above their optimum.

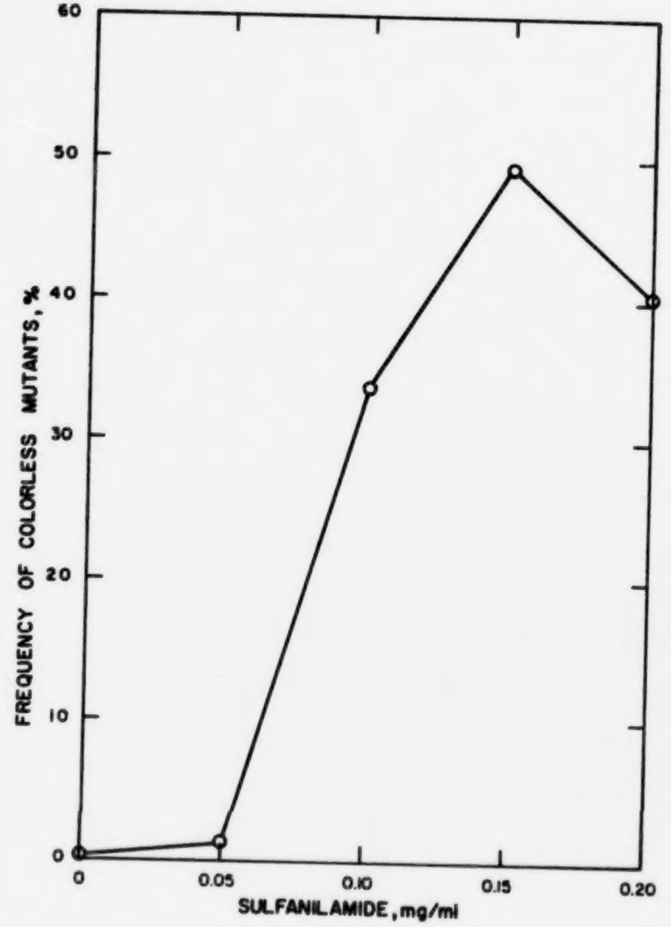

FIGURE 3. EFFECT OF SULFANILAMIDE CONCENTRATION ON THE FREQUENCY OF COLORLESS MUTANTS IN *Euglena*

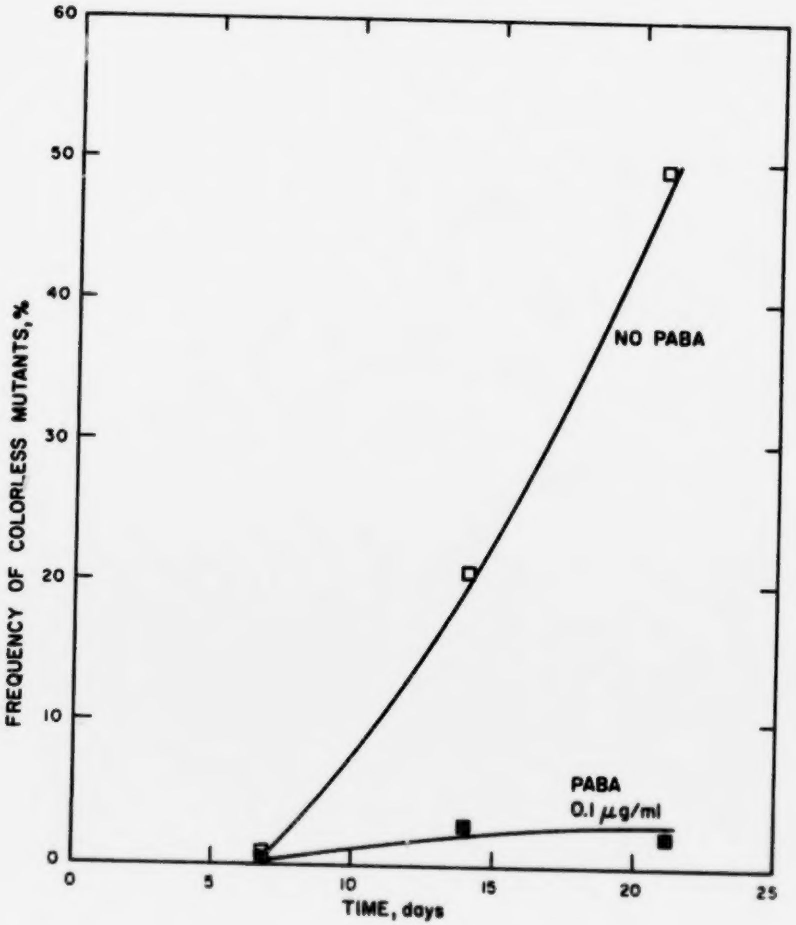

FIGURE 4. EFFECT OF p-AMINOBENZOIC ACID (PABA) ON THE FREQUENCY OF COLORLESS MUTANTS FORMED IN THE PRESENCE OF SULFANILAMIDE (0.15 mg/ml)

Experimental Conditions: As in Figure 2. After 3 weeks of incubation at 26-28°C samples were withdrawn for dilution and plating.

Quantitative Thermal Auxotrophy: Under permissive conditions, (below 33°C) *E. gracilis* exhibits requirements for thiamine and vitamin B_{12}. At slightly higher temperatures, the concentration of vitamins required to satisfy these preexisting nutritional deficiencies is increased by several order of magnitude over that needed under normal or permissive conditions. In Table IV we have recorded the results of experiments on the thiamine requirement in *Euglena* at permissive and restrictive temperatures. As the temperature is raised from 33.5 to 34.5°C increased amounts of thiamine are required for growth as compared to the control. Although the increment is small, the requirement for this vitamin is markedly increased at the higher temperature. Hutner *et al.* (1957, 1958b) and Scher and Aaronson (1958) have noted similar increases in the thermal requirements for vitamin B_{12} in *Ochromonas* and *Euglena*.

TABLE IV

EFFECT OF ELEVATED TEMPERATURE ON
THE THIAMINE REQUIREMENT OF
E. gracilis

Thiamine HCl	Temperature (°C)	
	33.5	34.5
None	1.1	0.2
1.0 μμg/mℓ	1.2	0.4
10.0 mμg/mℓ	1.4	0.4
0.1 mμg/mℓ	1.5	0.4
1.0 mμg/mℓ	1.7	0.6
10.0 mμg/mℓ	2.2	1.5
0.1 μg/mℓ	3.2	1.5
1.0 μg/mℓ	3.1	1.7

Experimental Conditions: The medium of Hutner *et al.* (1956) was modified as follows: Thiamine HCl was excluded; DL-lactic acid 0.4 mg/mℓ and glucose 10 mg/mℓ were added. Cell growth was determined as in Table I.

Qualitative Thermal Auxotrophy: Vitamins, amino acids, purines, and certain lipids not previously required for growth at normal temperatures, become essential, or stimulate growth at supraoptimal temperatures. Several examples can be cited: Addition of *p*-aminobenzoic acid is ineffective at 30°C, but overcomes growth inhibition at 34-35°C (Table V). Amino acids such as methionine, threonine, and tryptophan have little or no effect at 30°C, but appear to stimulate growth at higher temperatures (Table VI). Addition of adenine was also found to increase growth at 35.9 to 36.3°C but not at 30°C (Table VII).

TABLE V

EFFECT OF *p*-AMINOBENZOIC ACID ON THE GROWTH OF *Ochromonas malhamensis* AT ELEVATED TEMPERATURES

Temperature	*p*-Aminobenzoic Acid (mμg/mℓ)			
	0	1	3	10
30°C	2.2	2.3	2.9	2.7
34°C	1.0	1.6	1.8	1.8
35°C	0	0.5	1.3	1.8

Experimental Conditions: The basal medium (Table VIII) was supplemented with Thiamine HCl 1 μg/mℓ and vitamin B_{12} 0.1 mμg/mℓ. Cell growth was measured as in Table I.

TABLE VI

EFFECT OF AMINO ACIDS ON THE GROWTH OF *E. gracilis* AT ELEVATED TEMPERATURES

Amino Acid 0.05 mg/mℓ	Temperature °C		
	30	35.9	36.3
None	3.0	0.4	0
DL - Methionine	3.0	0.8	0
L - Threonine	3.0	0.8	0
DL - Tryptophan	3.0	0.8	0
DL - Methionine + L - Threonine	3.0	1.1	0
DL - Methionine + DL - Tryptophan	3.0	1.3	0.1
L - Threonine + DL - Tryptophan	3.0	1.3	0.1

Experimental Conditions: Cells of *E. gracilis* were grown in the medium of Hutner *et al.* (1956) supplemented with DL-lactic acid, 0.4 mg/mℓ; $MgSO_4 \cdot 7H_2O$, 1.0 mg/mℓ; vitamin B_{12}, 10 mμg/mℓ; L-proline, 40 μg/mℓ; DL-serine, 0.1 mg/mℓ; and DL-valine, 50 μg/mℓ. Growth was determined as in Table I.

TABLE VII

EFFECT OF ADENINE ON THE GROWTH OF *E. gracilis* AT ELEVATED TEMPERATURES

Adenine (μg/mℓ)	Temperature °C		
	30	35.9	36.3
0	3.0	0.4	0.2
20	3.0	1.1	0.9
40	3.0	2.1	1.7
80	3.0	2.7	2.0

Experimental Conditions: As in Table VI except that the medium was supplemented with a mixture of 17 amino acids (Table IX) replacing L-proline, DL-serine, and DL-valine.

At 37°C, nutritional deficiencies emerge for as yet unidentified growth factors (Baker *et al.* 1955). In contrast to the water soluble vitamins, amino acids, and purines, the unidentified factors required at higher temperatures may be due to blocks in lipid synthesis. Frank *et al.* (1960) reported that certain lipids permit growth of *Ochromonas danica* at 38°C. In the absence of these lipid factors, growth of *O. danica* does not occur above 35°C.

The requirements for specific growth factors that are not needed at normal temperatures but become essential at supraoptimal temperatures may be considered to be analogous to the analog-induced auxotrophy noted above. We therefore propose that thermal auxotrophy be designated a conditionally expressed characteristic which is revealed by cells exposed to restrictive temperatures.

TABLE VIII

BASAL MEDIUM FOR Ochromonas malhamensis

$(NH_4)_2H$ citrate	1.0 mg/mℓ
$CaCO_3$	0.14 mg/mℓ
$MgCO_3$ (basic)	0.5 mg/mℓ
L-glutamic acid	3.0 mg/mℓ
KH_2PO_4	0.3 mg/mℓ
DL-methionine	0.6 mg/mℓ
Sucrose	15.0 mg/mℓ
L-histidine HCl	1.0 mg/mℓ
L-arginine	1.0 mg/mℓ
Metals 45A[a]	0.01 mg/mℓ
$ZnSO_4 \cdot 7H_2O$	0.01 mg/mℓ
$Fe(NH_4)_2(SO_4)_2 6H_2O$	0.014 mg/mℓ
$MnSO_4H_2O$	1.0 μg/mℓ
Biotin	0.004 μg/mℓ

[a] For composition of metals 45A see Hutner *et al.* (1956).

TABLE IX

COMPOSITION OF AMINO ACID SUPPLEMENT

Amino Acid	Concentration mg/mℓ
DL-alanine	0.5
DL-aspartic acid	0.5
L-glutamic acid	0.8
Glycine	0.5
L-arginine	0.5
L-histidine (free base)	0.4
L-lysine	0.4
DL-isoleucine	0.05
DL-phenylalanine	0.04
DL-leucine	0.05
L-tyrosine	0.04
DL-methionine	0.05
DL-threonine	0.1
DL-tryptophan	0.04

Temperature-Induced Alteration of Indispensable Functions of Eucaryotic Organelles

Properties of the Temperature-Sensitive System: In Euglena, both replicative and transcriptive functions of chloroplast DNA proceed normally at temperatures below 27°C. When the temperature is increased above this level, a sensitive component of the chloroplast becomes altered. In contrast to temperature-induced auxotrophy which is reversible by addition of the product(s) of the blocked reaction, the temperature-sensitive component of the chloroplast cannot be by-passed nutritionally. Cells exposed to such restrictive temperatures produce mutant progeny that no longer harbor chloroplast DNA, and thus fail to develop a photosynthetic apparatus when returned to permissive conditions. The principal consequences of this mutational event are that: 1) the mutated cells can no longer depend upon light as a source of energy; 2) the cells are thus unable to utilize photosynthetic CO_2 fixation as a source of carbon; and 3) such cells become dependent upon an external supply of utilizable organic compounds, i.e., the mutation results in the transformation of a facultative photoautotroph into an obligate heterotroph.

Several properties of this temperature-sensitive component of chloroplasts have been investigated (Pringsheim and Pringsheim 1952; Brawerman and Chargaff 1960; Mego 1964b; Mego and Buetow 1965; Scher 1966) but little information is available concerning the range of temperatures that induce this mutation, or the kinetics of loss of chloroplasts at restrictive temperatures.

Kinetics of Chloroplast Loss at Restrictive Temperatures: To establish the range of temperatures over which chloroplasts of Euglena are inactivated, the following procedure was employed. Log phase, dark-grown cells were incubated at temperatures from 27° to 33°C; samples were withdrawn at suitable intervals, diluted to ~ 200 cells/0.1 mℓ, and plated on media that supported the growth of both wild type and chlorophyll-less mutants. After one week in light, to induce chloroplast development, the frequency of green and colorless colonies was determined.

At 25°C ± 1 the fraction of cells capable of forming green colonies remains constant. When the temperature is increased, the fraction of cells capable of forming green colonies decreases with time of exposure (Figure 5). After 72 hours at 33°C, less than 1% of the population retains the ability to develop green colonies when incubated under permissive conditions.

A computer program was used to determine if the kinetics of loss of chloroplast-forming ability conform to a simple dilution hypothesis. The available data do not precisely fit this model. The decay per generation appears to be greater than that expected from simple dilution. These results closely parallel the decay of photoreactivability following inactivation of chloroplasts with ultraviolet light (Hill et al. 1966).

Temperature-sensitive components of other eucaryotic organelles have also been reported: Growth of yeast at elevated temperatures induces the formation of petite mutants with defective mitochondria (Ycas 1956).

These temperature-sensitive characteristics observed in chloroplasts of Euglena and mitochrondria of yeast are analogous to certain temperature-sensitive mutants in bacteria. In these cases, the consequence of the thermal inactivation event is an irreversible mutation or deletion of a semiautonomous organelle. Such temperature-sensitive components of eucaryotic organelles detectable only under restrictive conditions may represent another class of nonauxotrophic conditionally-expressed characteristics.

RELEVANCE OF HETEROTROPHIC METABOLISM AND CONDITIONAL REQUIREMENTS TO EUTROPHICATION

Juday (1942) has estimated that the largest fraction of organic matter in natural waters is in solution, and thus available for heterotrophic metabolism. The evidence assembled here and in other more comprehensive reviews cited earlier supports the hypothesis that heterotrophic algal flagellates may respond to dissolved organic matter in several ways: Previous studies have demonstrated that organic compounds may stimulate growth by serving as auxiliary sources of carbon, nitrogen, and phosphorus, or as hydrogen donors for CO_2 fixation and nitrite reduction. We have presented evidence that external carbon compounds may exert control over the rates of biochemical syntheses through repression of enzyme formation or through feedback inhibition of enzyme function. The ability

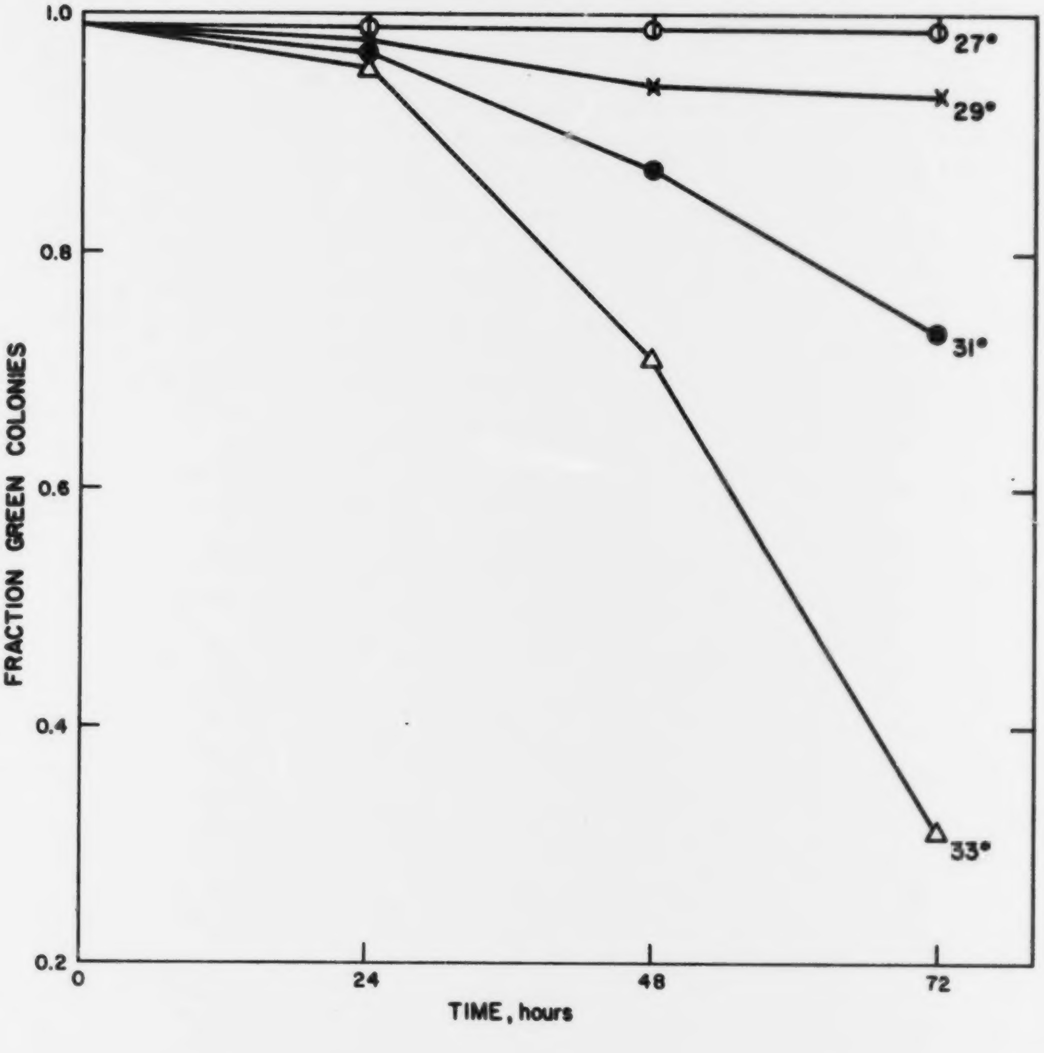

FIGURE 5. KINETICS OF LOSS OF CHLOROPLAST FORMING ABILITY IN *Euglena* AT SUPRAOPTIMAL TEMPERATURES

to utilize available carbon compounds to regulate cell metabolism may provide a selective advantage for heterotrophs over strict autotrophic strains.

Although conditionally-expressed requirements do not impose constraints under permissive conditions, they may prevent cell growth or irreversibly block organelle replication under restrictive conditions. Thus at supraoptimal temperatures, an increased need for thiamine or the emergence of a requirement for amino acids or purines may limit cell growth. Similarly, the inactivation of chloroplast replication at restrictive temperatures may lead to the loss of photosynthetic competence in a large fraction of the cell population.

Finally, we should recognize that in nature heterotrophic algae must compete for dissolved organic compounds with other heterotrophic microorganisms. Hobbie and Wright (1966) have compared the kinetics of substrate assimilation by bacteria and algae. They report that the rapid uptake of glucose and acetate by bacteria may limit the concentration of substrates available to algae. Kuentzel (1969) has called attention to the role played by bacteria in decomposing organic matter thus generating CO_2 for photosynthetic microorganisms in environments where CO_2 may be a limiting factor.

SUMMARY

The participation of dissolved organic compound in the heterotrophic metabolism of photosynthetic flagellates was examined. Physiological requirements for utilization of reduced carbon and nitrogen sources were determined and evidence for a regulatory function of exogenous metabolites in the control of biosynthetic enzymes was presented.

The paper calls attention to the use of cytochemical methods for isolating subcellular fractions, and selective metabolic analogs in the identification of latent or conditionally expressed auxotrophic requirements of eucaryotic cell organelles. An analysis of temperature-sensitive components was initiated, and two classes of thermally-induced defects were recognized. The significance of heterotrophic metabolism and conditionally-expressed requirements in eutrophication is briefly discussed.

ACKNOWLEDGMENTS

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PHYSIOLOGICAL ECOLOGY

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In the narrowest sense, physiological ecology is the study of the physiological responses of organisms to the variations in ecological conditions they experience in nature or to those to which they may be subjected experimentally in the laboratory. In the broadest construction, physiological ecology is that segment of biology that attempts to explain the natural distribution of organisms (i.e., why organisms live where they do). Traditionally, four approaches have been used to obtain this type of information: 1) Study of the geographic distribution of individual species (i.e., plant and animal geography) relating the range of distribution to the range in variation of specific ecological factors; 2) Analysis of variation in community structure along an ecological gradient, again equating the distribution, or the numbers of individuals of a species per unit area, to the intensity of expression of a factor; 3) Manipulation of simplified communities or species assemblages in laboratory model ecosystems or microcosms; and 4) Determination of the absolute requirements and range of tolerance for individual factors using unispecific cultures under completely artificial laboratory conditions.

At Berry Creek, the experimental controlled flow stream facility operated by the Pacific Cooperative Water Pollution and Fisheries Research Laboratories of Oregon State University, we have an example of an experimentally manipulated "natural" stream. It consists of four experimental sections; two "dark" sections with undisturbed canopy where the stream received approximately 40% of available light during la[illegible] spring, summer, and early fall; and two "light" sections in which the canopy had been thinned to admit 80% of available light. These sections were arranged in alternating series, dark, light, dark, light, and just above the second dark section a continuous addition of sugar solution was provided to maintain a concentration of 4 ppm in the lower "dark" and "light" sections. This arrangement could be considered either as a very abrupt "gradient" or as an experimental "treatment" and a "control."

When presented in graphic form, counts of algal cells colonizing glass slides (Phinney and Dever) kept immersed in each of the four sections illustrate the apparent effect of variations in light intensity, enrichment, and "season" on the algal biomass (Figure 1). Actually, we are aware that these data are accurate only in a relative sense. The summertime high counts are much lower than the chemical and physical conditions could produce. The reduction below the possible maximum is the result of herbivore activity. The "seasonal" low in winter is more a result of siltation than low temperature.

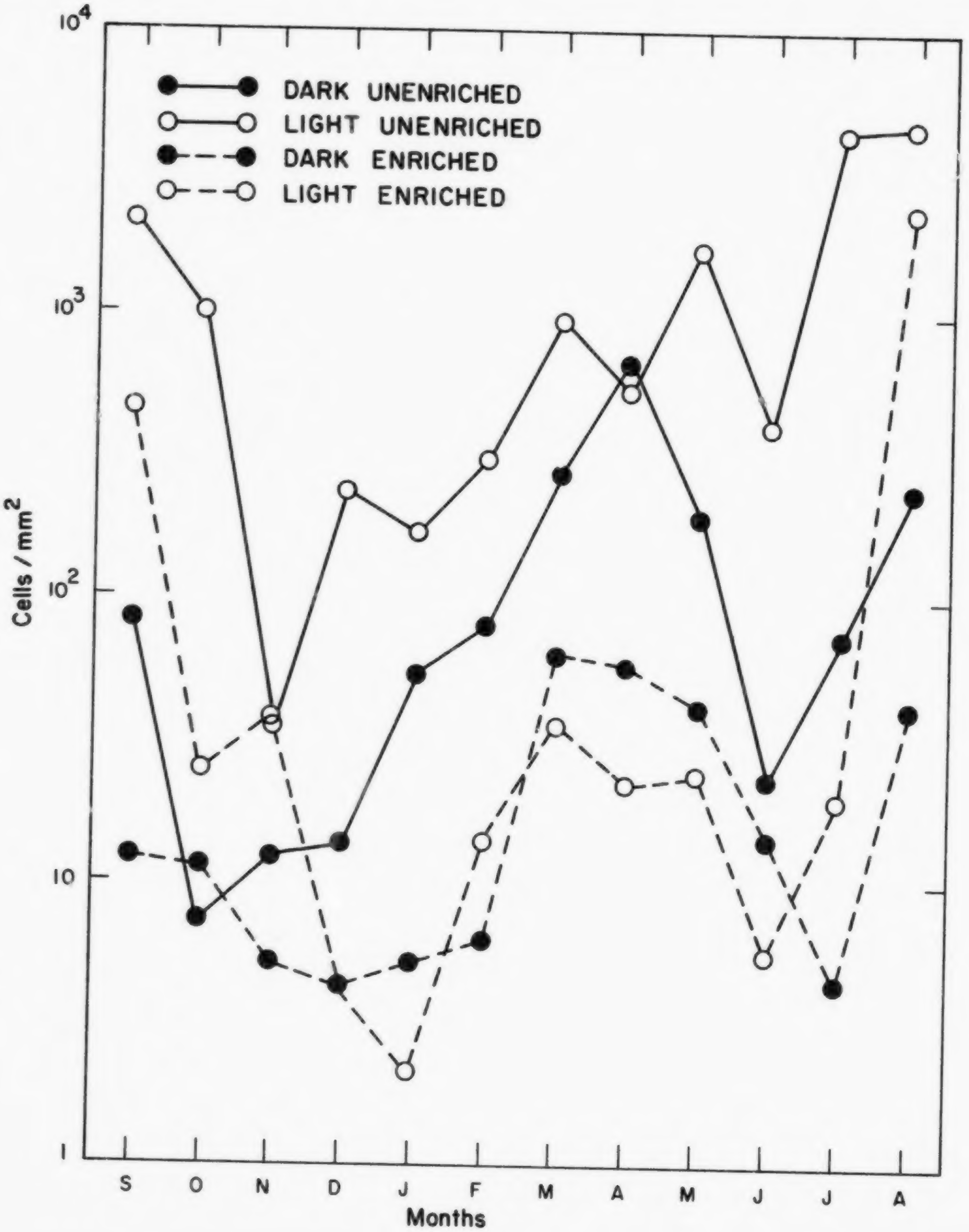

FIGURE 1. MONTHLY MEAN OF WEEKLY COUNTS, ALL ALGAE

The effects of a gradient in salinity on the distribution of macrophytes in Yaquina estuary have been studied (Kjeldsen 1967). It has been determined that there is a definite interaction of temperature with salinity that produces stresses that definitely limit the distribution of some species. The existence of interactions between ecological factors has been proven also in an autecological study of Aphanizomenon flos-aquae (O'Flaherty 1967). The experiments have shown significant interactions between several mineral ions, particularly in cultures maintained at higher light intensities. Sulfate-nitrate, sulfate-phosphate, sulfate-potassium, sulfate-iron, nitrate-bicarbonate, nitrate-zinc, phosphate-cobalt, phosphate-iron, etc. interactions were detected. Even a three-way interaction was documented between calcium, magnesium sulfate, and potassium monohydrogen phosphate using A. flos-aquae.

A study of the distribution of benthic algae in the Metolius River (Sherman 1969), a stream in which the velocity, temperature, turbidity, and water chemistry are remarkably stable, shows that a great majority of the species present are truly perennial and from a list of approximately 65 entities only 9 demonstrated any seasonal pattern of occurrence.

The structure of the benthic communities developed in laboratory streams under varying conditions of light intensity, current velocity, and time were analyzed by Dr. David McIntire (1968). He has reported a number of cases of interactions of light intensity and current velocity in affecting the structure of the community.

The basic objective of each of these approaches is to document, by direct or indirect evidence, the range of physiological responses of a species to the stresses created by variation in the intensity, quality, or timing of any of the environmental factors. Each approach has unique advantages and disadvantages with respect to the precision with which data can be obtained and each is subject to some criticism as to the general applicability of the information that the particular method yields. An impertinent statement of the general problem could go like this: the test-tube biologists criticize all field studies for their lack of controls and the near impossibility of making precise measurements; the natural-historian types criticize the laboratory models for being out of touch with reality. Both criticisms are valid to a point and a very vital activity in this type of research is the struggle to retain a balanced perspective and to be constantly aware of the pitfalls that await the investigator when he attempts to interpret the data and construct generalizations.

A favorite pastime of some physiological ecologists is attempting to identify particular species as indicators of specific conditions in the habitat. I view this as a futile occupation inasmuch as the distribution of a species is dictated by a complex of conditions. Significant variation in one or more of these conditions may give rise to physiological stresses, or conversely, relieve such stresses and cause changes in the relative abundance of the species of interest. Incautious interpretation by a careless investigator may well lead to the association of the change in numbers of individuals with changes in conditions that are prominent (season, temperature, light, etc.) or, by wishful thinking, to factors with special significance (pollution, low oxygen, high nitrate, etc.) that are in truth not causally related. It would seem unnecessary to point out that probably all of the algal species we deal with today evolved long before the time when man began to modify the environment. No one should be surprised if no species can be identified as an "indicator" of "pollution."

Perhaps the greatest danger of incorrect interpretation of results of ecological studies lies in the difficulty of separating the effects caused by long-term cyclic changes in environmental factors and effects resulting from noncyclic or at least short-term changes that may be superimposed on the long-term changes. As an example, let us consider the following situations: Changes in community structure and function may be attributed to "seasonal" effects. To be completely accurate, "seasonal" factors are those involving the intensity, duration, and quality of light that change with the changing season. Other nonseasonal factors, such as temperature, water quality, velocity of flow, turbidity that may be either cyclic or noncyclic, appear superimposed on the seasonal cycle and may produce changes in community structure interpreted as seasonal changes. This problem becomes particularly acute when considering environments under substantial influence of human activities. Many of man's activities are of a long-term cyclic nature and such human activities may cause changes in the environment that are in no way associated with truly seasonal factors.

Seasonal activity of animals other than man may effect changes in the environment that may be mistakenly identified as seasonal changes. Thus at one time we believed that the low biomass of periphyton of the woodland streams west of the Cascades in the summertime resulted from the greatly reduced light intensities available under the streamside canopies. When we began to operate model laboratory streams under light regimes comparable to those in nature, we found we developed extremely heavy periphyton growth in summer whose abundance apparently depended upon the absence of the herbivores native to the natural streams but which were absent in the laboratory models. We have found that introduction of these herbivores, primarily snails of the genus Oxytrema, will result in the almost total elimination of the periphyton communities in the model streams, just as the community is eliminated in the natural streams.

Another example may be warranted. Changes in the structure and function of the periphyton community may be attributed to a factor such as stream velocity. Depending upon the particular situation, changes in stream velocity may be long term or short term. If the changes are long term, the effects may be confused with the effects of other long-term factors, such as temperature, light, etc. If the changes in velocity result from short-term causes, the effects may still be confused with other concurrent and causally related factors such as changes in dissolved nutrients, turbidity, etc.

From these examples it is evident that the accumulation of information concerning the ecological control of species is slow, laborious, and fraught with danger of incorrect interpretation. These problems become even more acute when the research is a part of an effort to establish relationships between the structure and function of communities and conditions that usually prevail during culturally-produced eutrophication. Most commonly, such research is undertaken as a "crash" program. Often there is no opportunity to spend the time necessary to determine precise relationships or to make requisite comparisons or to carry out necessary experimental projects. Some of the studies qualify as descriptive natural history but often their contribution to either the understanding or solution of pollution are rather sketchy.

As a biologist, I have a strong interest in the study of ecological relations. I believe that it is imperative for mankind to have a very complete understanding of ecology and of the importance of preserving the environment. In order that the environment be protected we must know quite precisely what kinds of changes induce stresses in a community and what types of changes in structure will result.

However, I am convinced that to expect this kind of information to provide the guidelines that are presently hoped for is highly unrealistic. Much of the work presently being done under the guise of pollution research or water quality research is excellent biological research but has little or no prospect of producing anything that can be helpful in our hour of need. Do not misunderstand me, I think the work is very valuable and that it is highly important that it continue to be supported. However, it is improbable that such research will ever provide the solution to our present crisis in time to prevent the disaster many of us foresee.

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A LABORATORY APPROACH TO THE STUDY OF THE PHYSIOLOGICAL ECOLOGY OF BENTHIC ALGAL COMMUNITIES

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INTRODUCTION

W. D. Billings, the noted plant ecologist, has defined the field of physiological ecology as "the study of plant (and animal) processes under natural or simulated environmental conditions" (Billings 1960). Plant processes include, among others, photosynthesis, respiration, growth, germination, absorption, translocation, and reproduction; while some common animal processes are food consumption and assimilation, growth, respiration, movement, and reproduction. Some of my colleagues insist that there is no such field, that in reality it is all ecology, and I am not really sure when it became fashionable to speak of physiological ecology or physiological ecologists. However, I suspect that such terms had their origin a few years back when ecology was out of style, so to speak, suffering somewhat from a competitive interaction with the exciting and newer field of molecular biology. In any case, as biologists have come to realize the value of examining the results of physiological studies from an ecological point of view, an attempt has been made to recognize physiological ecology as a well-defined field of interest.

Billings' definition puts an emphasis on the natural, or at least a simulated natural, environment. With this in mind, let us briefly inspect the present status of algal ecology in terms of its potential contribution in the general area of water resource management. Many effects of changes in temperature and/or nutrient enrichment on the physical and chemical properties of natural waters are well known, but the influence of such changes on growth, metabolism, reproduction, and behavior of aquatic organisms is much less understood. In particular, studies of algae have been confined largely to the investigation of mineral requirements and the life history of selected species in culture. For example, of the 155 papers published in the last four volumes of the Journal of Phycology, only 23 (14.8%) reported the results of an analysis of data collected in natural or simulated natural environments and only 10 (6.5%) were concerned with a synecological problem. While culture studies have contributed much useful and valuable information, it has become increasingly apparent that many algae respond differently within a community unit, that is, when forced to compete and interact with other species, than in the simplified environment of the culture flask. Therefore, I contend that there is a significant gap in our knowledge of how populations of algae fluctuate within the complexities of natural communities. This certainly is not surprising, as such information is extremely hard to accumulate, and control over the environment usually is obtained at the expense of reality.

During the past decade new approaches [illegible] the study of ecological problems and new methods of processing data have been developed which provide ecologists with additional tools for dealing with the complexities of nature. One of the most promising new approaches has been the study of simplified communities in laboratory ecosystems, sometimes referred to as microcosms or microecosystems. Such systems, if designed properly, provide the investigator with some control over the environment, while at the same time, retaining many of the essential properties of natural ecosystems. Furthermore, the development of computer technology has made possible the employment of more complex statistical procedures which allow research workers to obtain considerable amounts of information from multivariable systems.

EXAMPLES OF LABORATORY ECOSYSTEMS

The beginning student of microcosm technology must be warned of the danger of becoming preoccupied with the elegance of his particular system. One can spend years doing nothing more than improving the design and perfecting various control devices. In fact, the scientific literature is now a graveyard of unused troughs, jars, and tubulations. Eventually the investigator must put down the hammer, saw, paint brush, and pipe wrench and get on with more important matters, i.e., the investigation of ecological problems.

For the past nine years, I have been involved in the design and use of laboratory ecosystems for study of the structure and function of benthic algal communities. The first design, a series of laboratory model streams, was constructed in 1959 at the Pacific Cooperative Water Pollution Laboratories, Oregon State University (see McIntire et al. 1964). These streams represented an attempt to model a riffle area of a natural stream. Water for the streams was supplied by a nearby spring-fed creek, and the algae were seeded naturally on the rubble and gravel substrate by organisms introduced through the water supply. A respirometer chamber also was constructed to aid in the measurement of community metabolism under different experimental conditions. More recently, a new laboratory system was constructed at the Oregon State University Marine Science Center, Newport, Oregon. This system consisted of a fiberglassed wooden trough, 3 m long, 76 cm wide, and 80 cm deep with the bottom graduated in a "stain-step" manner (McIntire and Wulff 1969). Tidal cycles were simulated by periodically pumping seawater into the system, and the illumination intensity was regulated by adjusting the height of a large lamp fixture over the trough. This system has been used to study the physiological ecology of marine benthic diatom communities.

From 1961 through 1965, productivity and bioenergetics of benthic algal communities were studied in six laboratory streams and a respirometer chamber. The objectives of these early experiments were to determine the effects of light intensity, carbon dioxide supply, dissolved oxygen concentration, temperature, and current velocity on rates of primary production, community respiration, and export of organic material. In general, it was found that an increase in the concentration of molecular carbon dioxide markedly increased the rate of photosynthesis of the algal communities at illumination intensities near and above 1,000 ft-c (Figure 1). At light intensities below 500 ft-c, a temperature increase of 10°C had no significant effect on photosynthetic rate, while at intensities above 1,000 ft-c the Q_{10} value varied between 1.3 and 1.6. Community respiration increased exponentially with increases of temperature in air-saturated water, but respiratory rates decreased rapidly as dissolved oxygen was reduced below air-saturation. Export of community material from the laboratory streams was closely associated

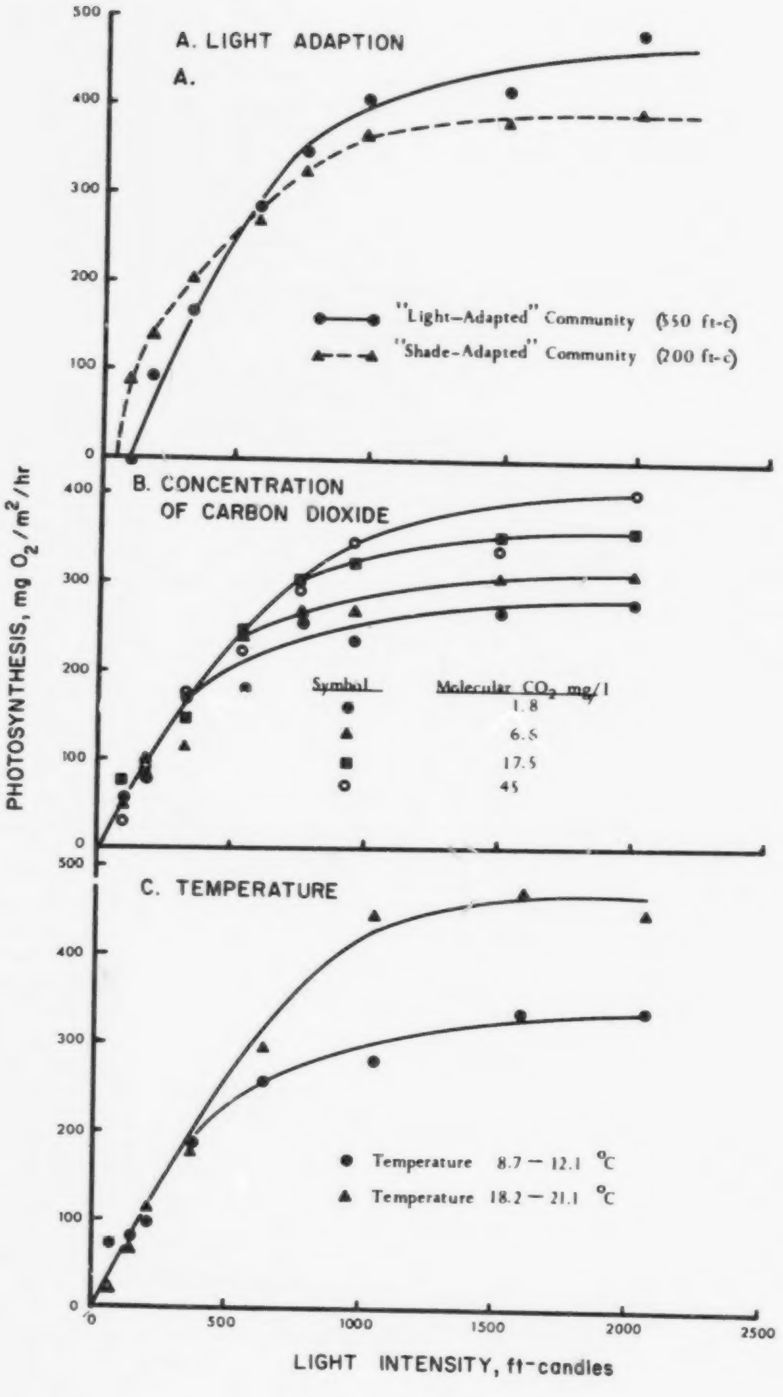

FIGURE I. THE RELATIONSHIP BETWEEN LIGHT INTENSITY AND RATE OF PHOTOSYNTHESIS FOR ALGAL COMMUNITIES DEVELOPED IN THE LABORATORY STREAMS

with biomass, current velocity, and the silt load of the water. When turbidity was low, rates of export were predicted by the equation

$$\hat{E} = b_1 B + b_2 (C \times B) , \tag{1}$$

where $\hat{E}$ was the predicted export (mg/24 hr), B was the biomass (g/m^2), C was the current velocity (cm/sec), and the b values were the partial regression coefficients associated with each variable. In the case of one set of data, the coefficient of determination (R^2) for Equation (1) was 0.75. For additional information concerning these experiments, the reader may refer to McIntire and Phinney (1965), Phinney and McIntire (1965), and McIntire (1966a, 1966b).

After concluding the experiments recapitulated above, it was still painfully obvious that we did not have a satisfactory understanding of even our small simplified microcosms. The fundamental problem was the paucity of information about the structure of the algal communities, particularly the species composition of the diatomaceous constituent. Unfortunately, synecologists usually have been either "productivity men" or "structure men," and there has been an understandable reluctance for field phycologists to deal with both aspects concurrently. Nevertheless, a simultaneous study of community structure and function became absolutely essential to the progress of our research.

The most serious barrier to the evaluation of species composition of communities of benthic algae in the laboratory streams was the lack of a satisfactory quantitative method for analyzing communities that consisted of complex mixtures of unicellular diatoms, filamentous diatoms, and filamentous chlorophytes, chrysophytes, and blue-green algae. Because of the filamentous nature and very small size of some common species, meaningful direct cell counts were difficult to obtain for the laboratory communities. As a result, a procedure based on the collection of "presence or absence" data was developed to overcome some of the difficulties imposed by the variety of growth forms (McIntire 1968). This method provided information that was related directly to the relative abundance of the algal species comprising the major part of the biomass of a community, but failed to evaluate quantitatively the distribution and abundance of rare species.

During 1966 an experiment was designed to obtain information concerning the effect of light intensity and current velocity on the relationship between the species composition and some selected biochemical and ecological properties of benthic algal communities in the laboratory streams. In contrast to earlier studies, emphasis was placed on a detailed analysis of species composition, with an attempt to obtain better understanding of how changes in certain physical factors regulated the relative abundance of the community constituents. Figure 2 illustrates the observed occurrence of Nitzschia linearis, a diatom, Phormidium retzii, a blue-green alga, and Tribonema minus, a filamentous xanthophycean, in six laboratory streams on seven sampling dates during the experiment. Occurrence represented the percentage of microscope fields in which a particular species was observed and was directly related to the relative abundance of the various algal taxa. Similar data were obtained for a total of 21 dominant species and were analyzed by fitting regression response surfaces which related occurrence to the experimental variables. The results of this analysis was related to other community properties such as biomass accumulation, export, pigment concentration, and fatty acid composition (McIntire 1968, 1969).

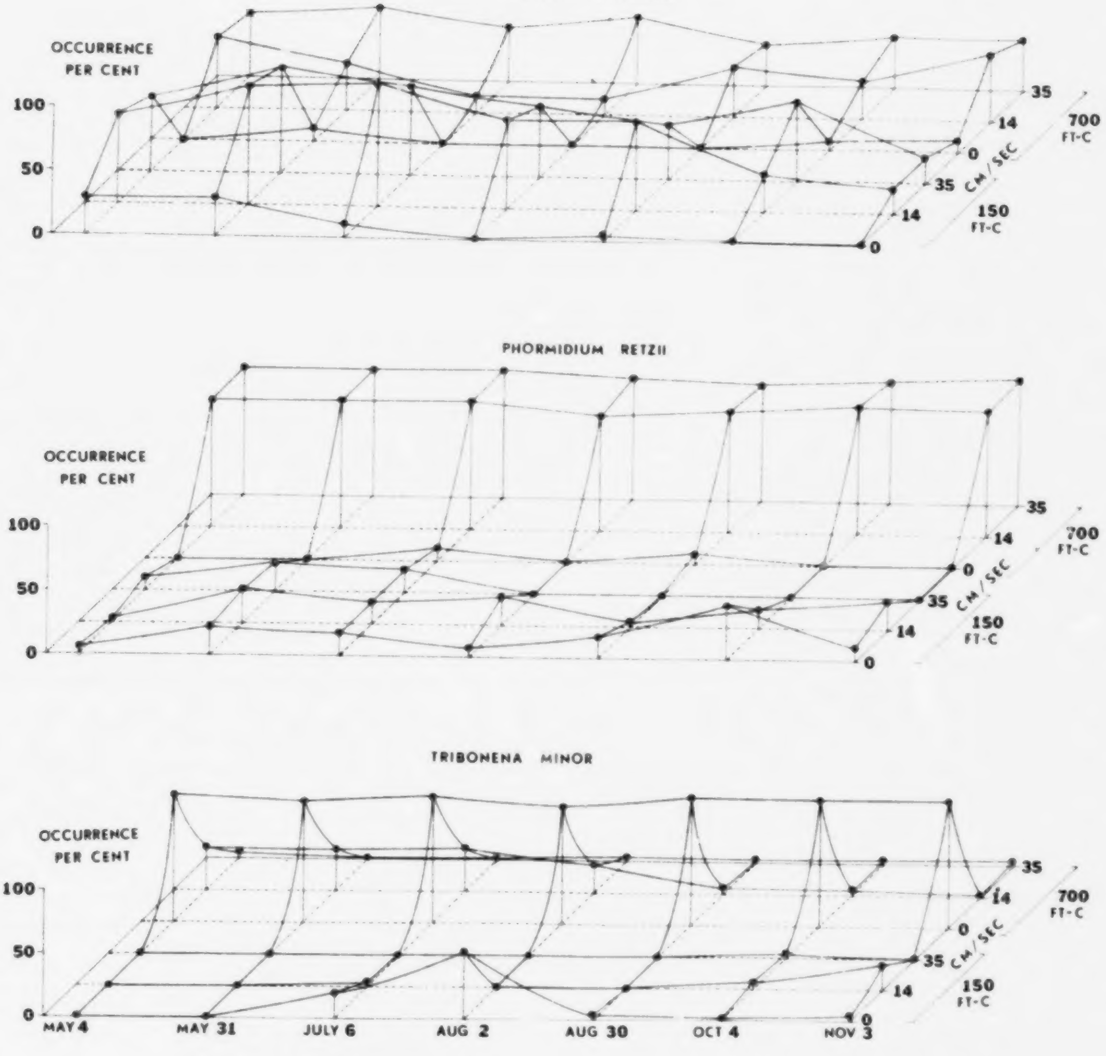

FIGURE 2. OBSERVED OCCURRENCE OF *Nitzschia linearis*, *Phormidium retzii*, AND *Tribonema minus* IN SIX LABORATORY STREAMS

An analysis of the residuals (i.e., the difference between the observed occurrence and that predicted from the regression equation) provided additional information about the communities. A matrix of correlation coefficients for the residuals of the 21 algal taxa suggested possible interaction between species, or at least the extent to which factors other than time, light intensity, and current velocity affected the covariance of all possible pairs of taxa. In all cases the coefficients were not particularly high, usually below 0.5, but distinct patterns of ecological interest were detected. For example, the residuals of seven diatom taxa exhibited a notable degree of positive correlation with each other, while several other diatoms apparently were more independent. The residuals of Phormidium retzii were negatively correlated with those of some diatoms, possibly indicating a competitive interaction. The correlation matrix served primarily as an exploratory technique that suggested probable species interactions. Additional experiments, both with unialgal cultures and whole communities in the laboratory streams, must be designed to gain a better understanding of these relationships.

In 1968 two experiments were performed using the marine laboratory system (Figure 3). Experiment TC-68 was designed to examine the effects of tidal cycle (period of exposure to desiccation and direct illumination) on the structure and productivity of diatom communities, while another experiment, LI-68, investigated the effect of light intensity on the communities. During experiment TC-68 the effluent stopcock was adjusted to such an opening that seawater would drain from a level of 10 cm above step 7 to the level of step 2 in 6 hr. After the 6-hr draining period, water was pumped back into the trough at a rate so that the influent water exceeded the effluent loss and refilled the system to its original level by the end of the next 6 hr. In experiment LI-68 the pump operated continuously to maintain water at a constant level of 10 cm above step 7.

Prior to the initiation of an experiment, one acrylic plastic plate was secured on each of the seven steps of the trough, beginning with step 1 at the deep end and progressing upward to step 7 at the shallow end. At the conclusion of each experiment, the plastic substrates with their established communities were transferred to a respirometer for measurements of community metabolism. Subsequently, the community material was loosened from the substrate with a rubber spatula and transferred into a bucket of distilled water. The total suspension volume was adjusted to two liters, and the sample was placed in a Waring blender for 20 sec. Subsamples were taken from this suspension for determination of biomass and species composition.

Measurements of biomass and community metabolism were made by Mr. Barry L. Wulff, Department of Botany, Oregon State University. The results of his work indicated that there was an inverse relationship between biomass accumulation and period of exposure to desiccation (experiment TC-68) and that biomass accumulation was the greatest on the substrate that received the highest light intensity (experiment LI-68). In experiment TC-68 slopes of curves relating illumination intensity to gross photosynthesis, expressed as oxygen evolution, were much steeper for communities from steps 1 and 2 than those from steps 3, 4, and 5. Biomass accumulation on substrates from steps 6 and 7 was negligible, and these substrates were not used in the respirometer studies. In experiment LI-68 the slope of the curve relating oxygen evolution to illumination intensity for the community from step 7 was much steeper than the corresponding slopes for the communities from steps 1, 3, and 5 at intensities between 5,000 and 18,500 lux. For more details of these experiments, the reader may refer to McIntire and Wulff (1969).

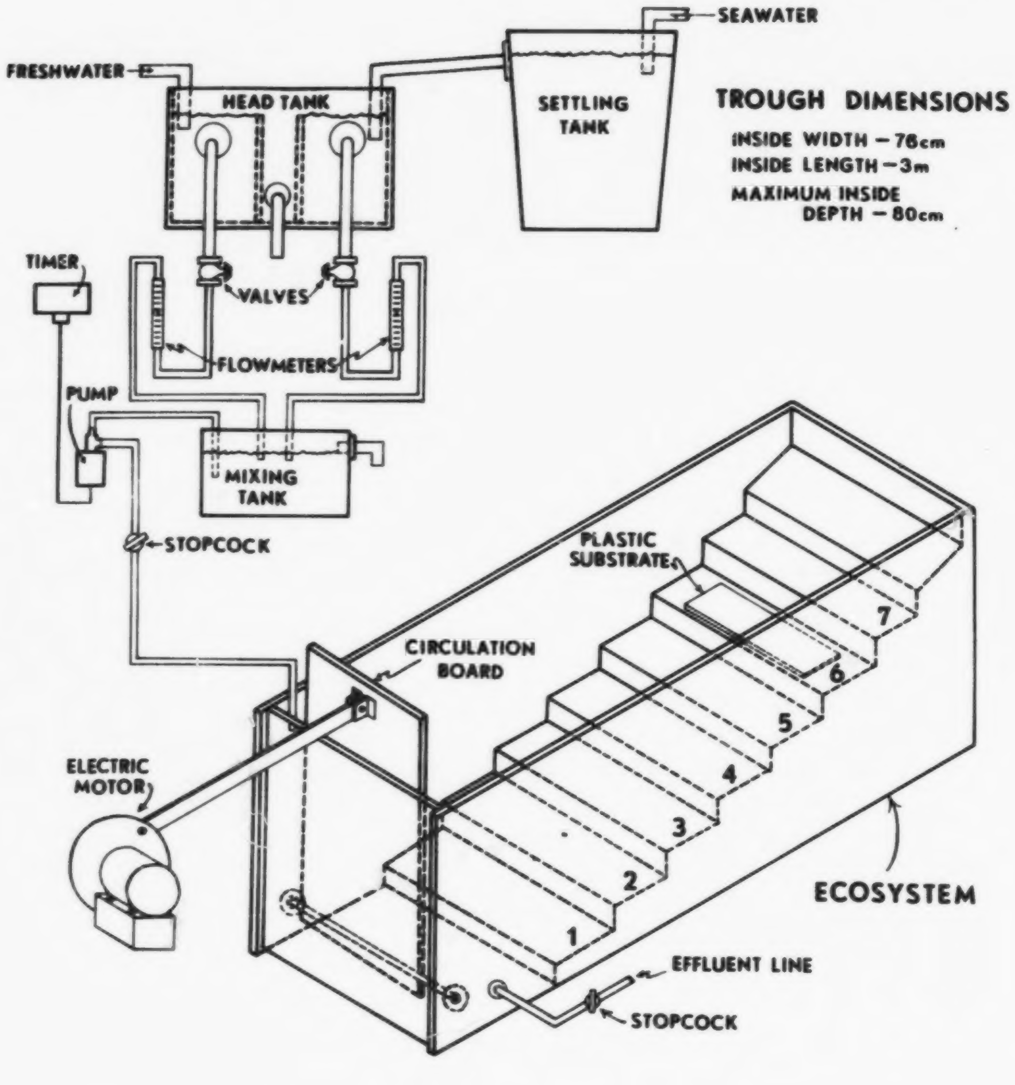
SEAWATER
FRESHWATER
HEAD TANK
SETTLING TANK
TROUGH DIMENSIONS
INSIDE WIDTH – 78cm
INSIDE LENGTH – 3m
MAXIMUM INSIDE DEPTH – 80cm
TIMER
VALVES
FLOWMETERS
PUMP
MIXING TANK
STOPCOCK
PLASTIC SUBSTRATE
CIRCULATION BOARD
ELECTRIC MOTOR
1
2
3
4
5
6
7
ECOSYSTEM
EFFLUENT LINE
STOPCOCK

FIGURE 3. MARINE LABORATORY SYSTEM

To quantitatively evaluate species composition of the diatom flora, approximately 1,000 diatoms were identified and counted for each sample under 1,250 X magnification. Vertical distribution of many species was closely related to light intensity and period of exposure to desiccation. For example, during experiment LI-68 Cocconeis scutellum var. parva represented 32.6% of the total number of cells on the substrate from step 1, the substrate that received the lowest light intensity (1,290 lux), but was considerably less abundant on the higher substrates (Figure 4). In contrast, three species, namely Fragilaria striatula var. californica, Amphipleura rutilans, and Melosira nummuloides, obviously competed best at the highest intensity (11,300 lux).

A convenient index to the degree of difference or "distance" between two communities is given by the equation

$$D_{jh} = \sqrt{\sum_{i=1}^{S} (P_{ij} - P_{ih})^2}\,, \tag{2}$$

where P_{ij} is the proportion of the i^{th} species in the j^{th} community, P_{ih} is the proportion of the i^{th} species in the h^{th} community, S is the number of species, and D_{jh} is the distance between the j^{th} and h^{th} communities. In other words, a small D_{jh} value indicates that the two communities are similar with respect to species composition and the relative abundance of the species.

Table I gives a matrix of distance values for comparisons of communities sampled in experiments TC-68 and LI-68. The first two letters indicate the experiment, and the number designates the particular substrate. The data are separated into three blocks, the upper left block compares communities within experiment TC-68, the lower right block compares communities within experiment LI-68, and the upper right block compares communities among the two experiments.

Communities TC:3 and TC:5 and communities LI:3 and LI:5 were the two most similar pairs, while community LI:7 was the most unique in species composition. Communities TC:3 and TC:5 were both exposed to periods of desiccation, 1 and 8 hr/day, respectively; while LI:3 and LI:5 were exposed to intermediate light intensities (Figure 4). Community LI:7 had the lowest diversity, and Fragilaria striatula var. californica was 51.8% of the community, the highest percentage recorded for a single taxon during either experiment. The distance values for all possible comparisons between experiments did not vary greatly and were relatively large. This was probably the result of differences in experimental conditions as well as seasonal differences in the diatom flora of the estuary.

Our experiments have demonstrated the potential of laboratory ecosystems as another tool for the investigation of aquatic communities. However, all such systems result from simplifications of nature, and some reality is usually sacrificed in the process of gaining control over the environment. In my opinion, laboratory ecosystems are best used to gain information that can supplement and help in understanding concurrent observations in the field.

ORGANISM IDENTIFICATION

Cocconeis scutellum var. *parva*
Nitzschia sp. #1
Synedra fasciculata
Thalassionema nitzschioides
Cocconeis costata
Bacillaria paxillifer
Nitzschia longissima
Nitzschia sp. #2
Fragilaria striatula var. *californica*
Amphipleura rutilans
Melosira nummuloides

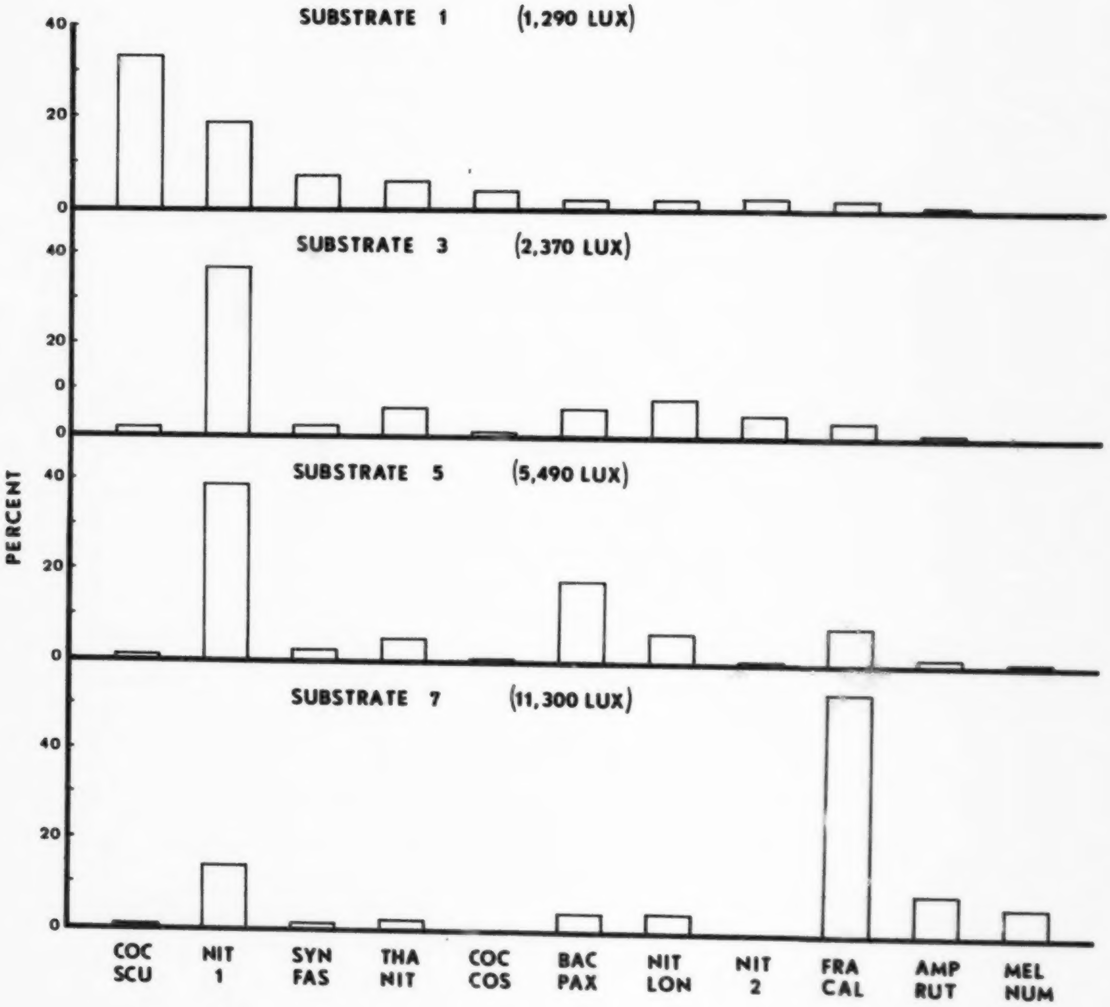

FIGURE 4. RELATIVE ABUNDANCE OF THE FIVE MOST ABUNDANT DIATOM TAXA ON STEPS 1, 3, 5, & 7 AT THE CONCLUSION OF EXPERIMENT LI-68

TABLE I

MATRIX OF DISTANCE VALUES FOR COMMUNITIES SAMPLED IN EXPERIMENTS TC-68 AND LI-68

	TC:1	TC:3	TC:5	LI:1	LI:3	LI:5	LI:7
TC:1	--	3.08	3.43	5.03	5.09	5.39	6.44
TC:3		--	1.09	4.50	4.50	4.80	5.83
TC:5			--	4.56	4.53	4.88	5.80
LI:1				--	3.83	4.23	6.16
LI:3					--	1.41	5.61
LI:5						--	5.37
LI:7							--

NOTE: A matrix of distance values (D_{jh}) for comparisons of communities sampled during experiments TC-68 and LI-68 where

$$D_{jh} = \sqrt{\sum_{i=1}^{S} (P_{ij} - P_{ih})^2} .$$

The values in the table are $D_{jh} \times 10^1$.

CONCLUDING REMARKS

Quite frankly, I feel that we are not training enough systems-oriented ecologists with specialized knowledge of the algae. We need ecologists equipped with the "new tools," individuals who are prepared to contend with the complexities of natural communities. We need ecologists with an interest in ecosystem structure, function, and evolution. We need ecologists who can communicate with both politicians and molecular biologists! In short, we need to reevaluate our educational program in terms of relevancy to the most urgent problems of environmental protection. Unfortunately, environmental biologists are spinning their wheels. They are in danger of becoming obsolete while the engineers are taking over their function.

And how does phycology compare to other areas of the biological sciences in this "turtle race" for quick answers to complex environmental questions? For almost 100 years the phycologist has retreated to the confines of the culture flask for these answers. Consequently, there is now a sacred myth, perpetuated and widely supported by many phycologists, that experiments with multiple species systems and observations in the field where variables are uncontrolled are somewhat tainted, and that the only productive approach is to start with a culture of an alga (it must be bacteria free) and after a series of rigidly controlled experiments, formulate inferences about the organism's role in nature. I must state at this point that it is not my intention to minimize the efforts of many competent scientists who have contributed valuable knowledge concerning the physiology, biochemistry, and life history of many algal taxa by studying individual species in culture. But let us take a close look at the basket in which we are putting all the eggs (i.e., the culture flask). To begin with, most culture media are extremely rich in the minerals essential for algal growth, and these high concentrations are present during the exponential phase of growth. For example, the recommended algal nutrient medium for the Provisional Algal Assay Procedure includes 14 mg/ℓ nitrogen and 0.62 mg/ℓ phosphorus, concentrations considerably higher than those found in most natural environments, even when eutrophication is a serious problem. Furthermore, the species diversity in an axenic algal culture is zero because $-\Sigma p_i \log p_i = \log 1 = 0$, a fact frequently ignored. Many assemblages of algae in nature consist of over 100 species of diatoms alone, and species diversity, expressed by the same index, often ranges as high as four. Also, there is the matter of light quality and intensity. Most cultures are grown under fluorescent lamps at intensities of about 200 ft-c, while in nature intensities often approach 10,000 ft-c and the red end of the spectrum is better represented. Finally, a time period that is ecological history for higher plants and animals can be evolutionary history for microorganisms. Therefore after a few years in culture, an organism becomes better adapted genetically to culture conditions and changes its ecological properties.

In summary, my plea is for a balanced attack on algae-related problems in the environment. To balance the scales, we need a shift in emphasis from the culture flask to ecological investigations in natural or simulated natural environments. This shift must be made by biologists, not engineers, and by biologists familiar with the newer statistical techniques and with a good background in theoretical ecology and the principles of resource management. The field biologist is in contact with reality. He knows the problems and the important questions to ask. He must communicate with the physiologists, the culture men if you like, so that they will help him answer the relevant questions.

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LIMNOLOGICAL INVESTIGATIONS SESSION

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Corvallis, Oregon

^{14}C UPTAKE AS A SENSITIVE MEASURE OF THE GROWTH OF ALGAL CULTURES

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INTRODUCTION

Since the ^{14}C technique was first introduced by Steemann-Nielsen (1951, 1952), it has become increasingly popular with oceanographers and limnologists wishing to estimate the rate of carbon fixation in natural waters. In the ^{14}C technique, a small amount of radioactive $^{14}CO_2$ is added to an algal culture. After several hours of incubation, the algae are collected on a membrane filter and the amount of radioactive carbon taken up by the algae is measured by counting the radioactivity on the filters by means of a Geiger-Mueller counter. (See Goldman 1963, for details of procedure.)

Even while this technique was gaining a wide acceptance, there was a controversy among its users about whether gross or net productivity, or something in between, was being measured. This controversy has now been resolved to the extent that it is possible to say that "Where excretion (of newly labeled compounds) is insignificant, the carbon-14 method gives a measure of net photosynthesis," (Bunt 1965). The experimental evidence supporting this claim indicates that algae conserve their respiratory CO$_2$, feeding it back into the photosynthetic pathway rather than releasing it to the environment. Excretion of newly labeled compounds is, however, a frequently observed phenomenon. Thus most authors now take the view of McAllister, Shah, and Strickland (1964) that the measurement represents increases in particulate carbon. The method has undergone considerable modification and improvement, and problems of calibration have been remedied (Goldman 1960, 1963, 1968).

There have been several experiments demonstrating the equivalence of ^{14}C uptake and increases in particulate organic carbon. Strickland's group at Nanaimo, British Columbia, studied the increase in particulate organic carbon relative to ^{14}C uptake by sampling a culture of natural phytoplankton kept in a large transparent plastic sphere submerged in Departure Bay. With daily ^{14}C measurements and daily estimates of algal biomass they were able to demonstrate a close correspondence between these two indicators of cell growth. The slight discrepancy that appeared after 18 days could be accounted for by the excretion of labeled compounds, (Antia et al. 1963).

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Ryther and Menzel (1965) performed laboratory experiments which gave further evidence that the ^{14}C technique measures the increase of particulate organic carbon. The averages of 37 particulate and ^{14}C values were 5.98 and 5.95 mg C/ℓ, respectively.

The ^{14}C technique has also been modified for use in bioassay experiments in which some suspected limiting nutrient is added to an algal culture and its effect on algal growth is measured in terms of the ^{14}C taken up by the cells (Goldman 1960). This type of experiment is an alternative to determination of the absolute value of the photosynthetic rate and requires less time. It is not necessary to determine the efficiency of the counting equipment nor to know the ratio of ^{14}C to ^{12}C in the culture medium as long as carbon does not become limiting in the environments. Experimental results can be expressed in terms of their deviation (in cpm) from the control. This approach has been used extensively in evaluating the eutrophicating effects of nutrients entering Lake Tahoe from its tributary streams (Goldman 1964; Goldman and Armstrong 1969). Other methods employed for measuring growth in cultures include making cell counts, measuring the increase in light extinction as the cells multiply, and determining the biomass of cells produced. When bioassays are performed with dense unialgal cultures these methods give comparable results. This report summarizes the results of experiments designed to compare the reliability of ^{14}C carbon experiments with these other methods.

The experiments were carried out through a joint effort of personnel attached to the FWPCA Research Project (USDI 16010DBU) and the FWPCA Demonstration Grant Project (WPD-48) at Lake Tahoe. Special thanks are due Professors Pearson, McGauhey, and Hasler for encouraging the direct comparison of growth measures between the two projects.

METHODS

A culture of Chlorella pyrenoidosa was established by taking algae from an agar plate and inoculating them into a liter of Bristol's solution buffered with 2.5 g $NaHCO_3$. After 24 hours, the culture was diluted to 2 liters with Bristol's solution, mixed well, and divided into two large flasks. Then ^{14}C was added directly to one of the large flasks. The other large flask was left unlabeled so that ^{14}C could be added to subsamples taken at 24-hour intervals. These subcultures were incubated for 4 hours after which 25-mℓ samples were collected on HA Millipore filters for counting of radioactivity. Fifty-mℓ subsamples were taken from the large labeled culture at 24-hour intervals also. Each of the two large cultures (one labeled with ^{14}C and one unlabeled) was sampled for cell counts and for optical density measurements at 600 mμ. The subculturing and sampling routine was repeated at 24-hour intervals over the next 4 days. After the second sampling, the lights were accidentally turned off for 24 hours. Lights were turned on for the third subculture and remained on for the rest of the experiment.

In a second series of experiments, filtered secondary effluent from the Reno-Sparks Sanitary Treatment Plant and filtered water from the Upper Truckee-Trout Creek were added as a biostimulant at 1%, 10%, and 50% concentrations to filtered Lake Tahoe water. An equal inoculum of algae, Selenastrum gracile (I. U. 325) was added to 250-mℓ flasks, each containing 160 mℓ of media. On the 2nd, 4th, and 6th day, growth was measured in three flasks of each treatment in the following manner: 10 mℓ was removed for cell count, 50 mℓ for alkalinity titration and pH measurement, and 100 mℓ was inoculated with ^{14}C for a total of 160 mℓ . Cell count, pH, and alkalinity were performed on the water removed from the sample just before the 3-hour ^{14}C incubation. The flasks were covered with air-tight rubber snap-caps. One of the three flasks of each treatment was covered with aluminum foil and was retained as a dark control for the measure of nonphotosynthetic carbon assimilation. After 3 hours, under the same light at which they had been incubated, 50 mℓ from each flask was filtered on HA Millipore filters for the measurement of radioactivity. This procedure gave cell count and alkalinities from 3 replicate flasks for each concentration and each date. Each flask was individually marked so that total carbon values and ^{14}C uptake for each could be calculated as the mg of carbon assimilated per cubic meter per 24-hour day of constant light.

A third type of experiment utilized the natural phytoplankton populations resident in Lake Tahoe. Lake water was collected from 5 m depth 1 mile from shore and 500-mℓ samples were measured into separate Erlenmeyer flasks. Water taken from the mouth of the Upper Truckee-Trout Creek was added to the flasks at ratios of 0:1 (control), 1:1000 (0.1%), 1:100 (1.0%), and 1:10 (10%). Ten μc of ^{14}C as sodium carbonate were added to each flask and the flasks were placed in a special incubator designed to give identical lighting to each flask. At 24-hour intervals thereafter, for 5 days, 50-mℓ aliquots were withdrawn and filtered onto HA Millipore filters for radioactivity determinations. The total inorganic carbon concentration was determined by alkalinity titrations of the lake water and of the mixtures of lake and stream water. The radioactivity values for the cultures were corrected for any differences in total inorganic carbon, so that they could be compared directly with each other. The phytoplankton were identified and counted in the original lake water sample and in the cultures after 5 days of growth.

Another experiment of this third type tested the effect of additions of mineral nutrients. Offshore epilimnetic water was again used as the culture medium. Experimental treatments included adding nitrogen at the rate of 6 and 24 ppb, NO_3-N and phosphorus at the rate of 3 and 9 ppb PO_4-P. Nutrients were added singly and in all possible combinations so that P/N interactions could be detected. ^{14}C was added to an 18-liter carboy of surface water and then portions were withdrawn for the nutrient additions. This procedure assured that all treated flasks as well as the controls had equal dosages of ^{14}C. The 500-mℓ flasks were incubated under natural lighting for 4 days and 50-mℓ subsamples were removed approximately every 24 hours. A similar experiment tested the effect of additions of nitrogen and iron both singly and in combination. Rates of additions were 0, 5, and 20 ppb NO_3-N and 0 and 5 ppb Fe as iron citrate.

RESULTS

The results of the Chlorella experiments are shown in Figure 1. The Chlorella culture was established by inoculating the media with a small number of algal cells, so that it was not until the third or fourth sampling period that the cells reached log growth. Only the beginning of the log growth is shown. We know by previous experiments that the maximum cell density of Chlorella in Bristol's solution is much higher than the density reached in this experiment.

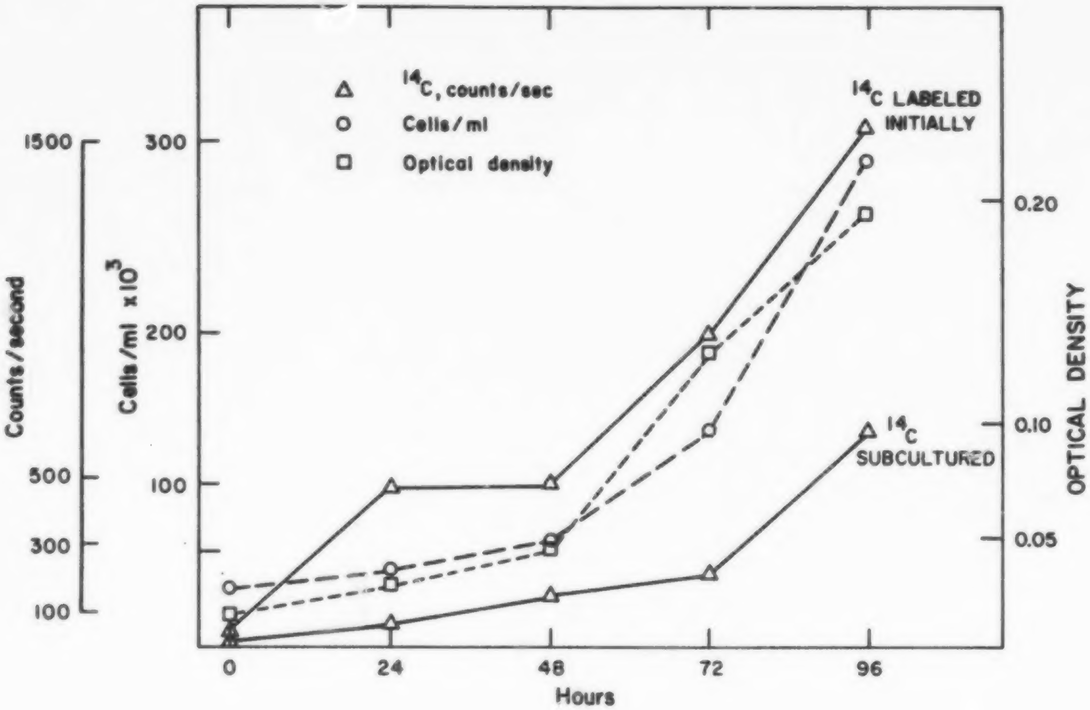

FIGURE I. RESULTS OF FOUR METHODS OF ASSAYING GROWTH IN A *Chlorella* CULTURE OVER A 4-DAY PERIOD

NOTE: The short dashed line indicates the optical density of the culture at different times during the 4 days. The long dashed line indicates the density of cells. The lower solid line indicates the instantaneous growth rate as measured by radiocarbon on each of 4 days and the upper solid line indicates the total amount of radiocarbon assimilated during the experiment. The culture was in darkness between the 24th and 48th hour. Illumination was continuous for the rest of the experiment.

Figure 1 shows the change with time of mean cell count and optical density of Chlorella cells in two cultures. The cell counts and optical density values from the labeled and unlabeled flasks were viewed as replicate determinations, the only difference between the cultures being the addition of a small amount of radioactive ^{14}C to the labeled flask.

The ^{14}C counts/second in the figure are from radioactive filter counts taken from the labeled flasks and from the ^{14}C cultures started from subsamples of the unlabeled flask. The ^{14}C counts/second from the labeled flasks give a measure of the total amount of carbon taken up by the algal cells from the beginning of the experiment. The ^{14}C subculture counts/second measure the amount of carbon taken up by the cells in 4 hours. The latter counts are, of course, always much lower than the counts in the labeled flask.

The results of the experiments using Reno Sewage Treatment Plant effluent and Upper Truckee-Trout Creek water are shown in Figures 2 and 3. These figures show carbon fixation for 3 hours, the total amount of carbon fixed after 2, 4, and 6 days, and the cell counts determined at the same time as the radioactive

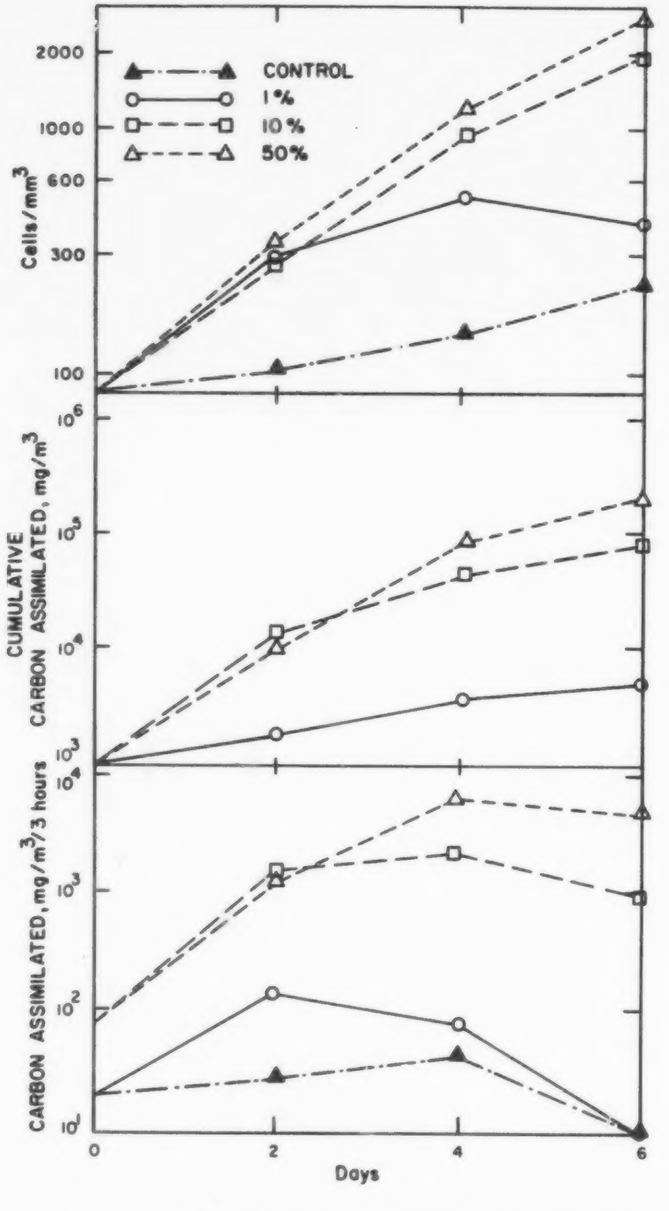

FIGURE 2. RESULTS OF BIOASSAYS OF RENO SECONDARY TREATMENT PLANT EFFLUENT-^{14}C AND CELL COUNT METHODS

NOTE: Effluent was added in the amounts indicated to cell-free Lake Tahoe water and a standard inoculum of Selenestrum gracile was added to each flask. The bottom panel gives the instantaneous growth rates measured at different times during the 6-day period. The center panel shows the same data rearranged on the assumption that the average rate of carbon assimilation over a 48-hour period was the average of the instantaneous rate at the beginning of the period and at the end of the period. The upper panel shows the measured cell densities at different times during the period.

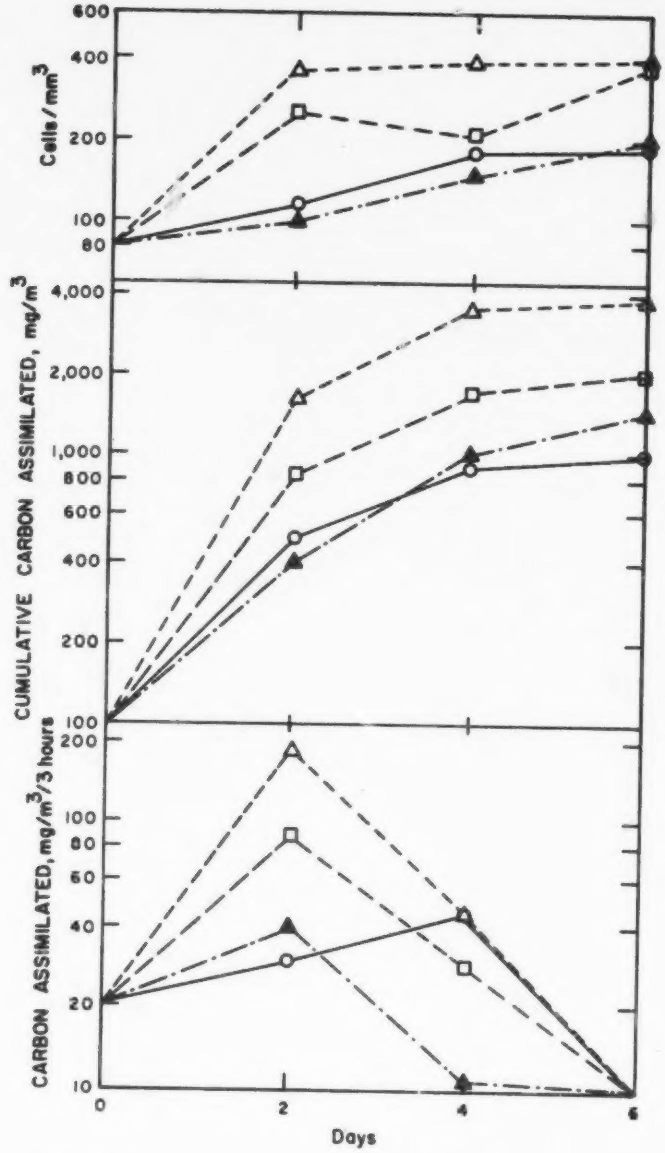

FIGURE 3. RESULTS OF BIOASSAYS OF UPPER TRUCKEE-TROUT CREEK WATER-^{14}C AND CELL COUNT METHODS

NOTE: Except for the source of the material being assayed, the experiment was in every detail like that shown in Figure 2.

uptake experiments were conducted. Total cumulative carbon fixed and cell count should be directly comparable to each other as they both give a measure of the particulate material fixed up to the date of the experiment. The cell count values appear not to be as reliable as the cumulative carbon fixation figures. Note the decrease in cell number on the fourth day of the 10% flasks utilizing Upper Truckee-Trout Creek water and on the sixth day of the 1% flasks utilizing Reno Sewage Treatment Plant effluent. There is no explanation for a loss of cells in the cultures on those dates.

Figure 4 and Table I show the results of the experiment with Lake Tahoe water containing indigenous algae at their natural population density. Growth stimulation was provided by the addition of filtered Upper Truckee-Trout Creek water in the proportions noted earlier (0.0%, 0.1%, 1.0%, 10.0%). The results in Figure 4 are given simply as counts per minute and express the

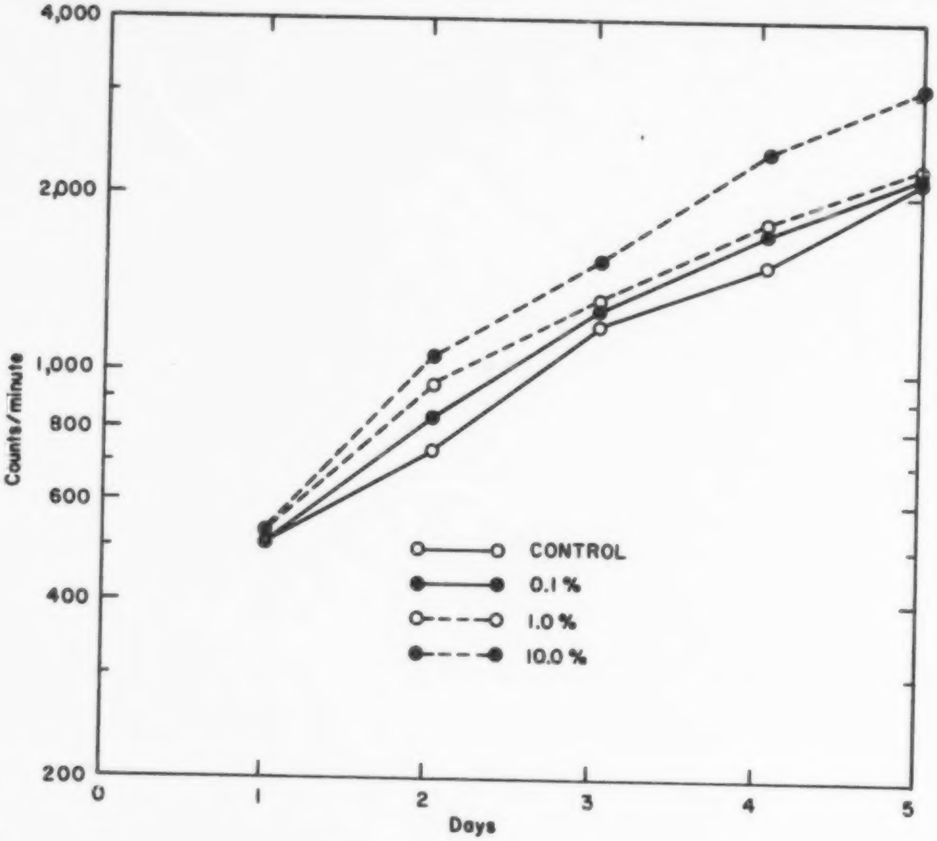

FIGURE 4. BIOASSAY OF UPPER TRUCKEE-TROUT CREEK WATER-^{14}C METHOD

NOTE: In this experiment the culture medium consisted of raw Lake Tahoe water containing its resident phytoplankton community at normal cell densities. Stream water was added in the proportions indicated and the cumulative amount of ^{14}C taken up was measured each day for 5 days.

cumulative incorporation of ^{14}C over a period of 5 days. Those for the 10% addition of stream water have been corrected for the reduced concentration of inorganic carbon in the culture. After the first day, the counts per minute in the samples filtered from the 0.1, 1.0, and 10.0% treatments were higher than those taken from the control; the counts per minute from the 10% flasks being more than 1000 counts per minute higher than the control.

Table I lists the phytoplankton counted in this experiment initially and at the end of 5 days of incubation. The figures given for the initial count would be less in the flasks where the Lake Tahoe water was diluted by creek water, but in looking at the final cell counts it is not possible to determine with any certainty that there has been any cell growth in any of the flasks. The only certain result was that the Chlorophyceae did not survive the 5 days of incubation. The initial count is for the algal cells in the undiluted Lake Tahoe water. The obvious differences between treatments detected by the ^{14}C method did not influence the cell counts.

TABLE I

PHYTOPLANKTON COUNTED

Time of Count	Conc. of Biostimulant cells/mℓ	Algal Class				
		Bacillario-phyceae	Chloro-phyceae	Cyano-phyceae	Chryso-phyceae	Dino-phyceae
Initial	0.0%	1334	192	0	40	30
After 5 days Incubation	0.0	1944	0	0	24	20
	0.1	2008	2	0	124	20
	1.0	1862	0	0	74	26
	10.0	1848	0	0	96	6

NOTE: Cell count of the natural phytoplankton in Lake Tahoe water and upon the addition of filtered Upper Truckee-Trout Creek water, initially and after 5 days of incubation. The creek water was added at 0.1, 1.0, and 10.0% concentrations by volume.

Figure 5 shows the results of nitrogen and phosphorus additions to Tahoe surface water. Low addition of nitrogen (6 ppb NO_3-N) had a slight stimulatory effect while a greater addition (24 ppb) had a marked inhibitory effect. Addition of phosphorus by itself had no effect either at 3 ppb PO_4-P or 9 ppb. The combined effect of nitrogen and phosphorus was to stimulate photosynthesis; the greatest stimulation resulting from the maximum addition of the two nutrients.

Iron deficiency for phytoplankton growth in Tahoe was first detected in 1960 (Goldman 1964). Figure 6 shows the results of iron and nitrogen additions to Tahoe surface water. Nitrogen at 5 ppb NO_3-N had no effect and only slight stimulatory effect at 20 ppb. Iron alone at 5 ppb had a marked stimulatory effect and this effect was enhanced further by the addition of nitrogen. Maximum stimulation was achieved with 5 ppb of iron as iron citrate added together with 20 ppb NO_3-N. Note that after four days, net growth in these cultures had ceased.

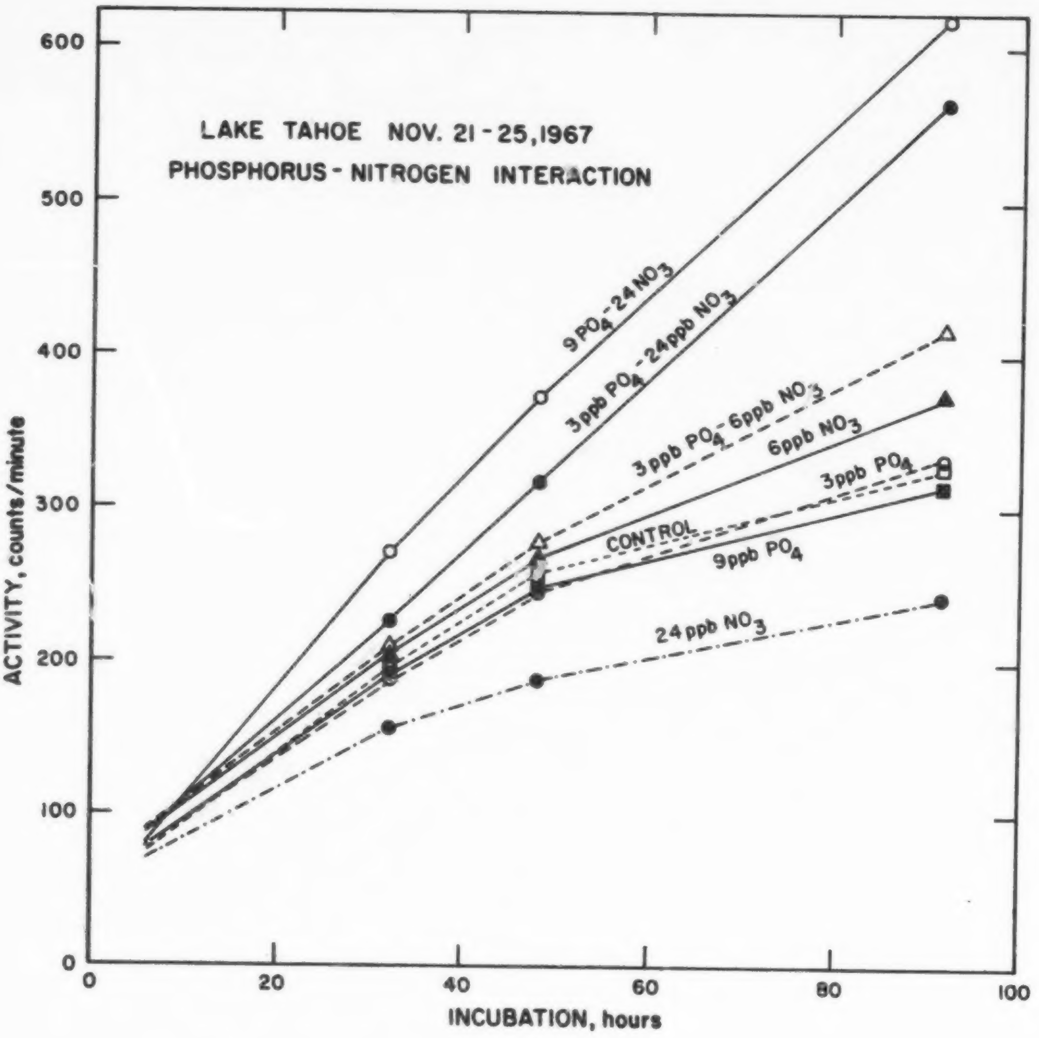

FIGURE 5. RESULTS OF NITROGEN AND PHOSPHORUS ADDITIONS TO LAKE TAHOE SURFACE WATERS

NOTE: The elements were added as dilute solutions of their sodium salts. Neither element had an appreciable stimulatory effect alone but when added together there was marked stimulation.

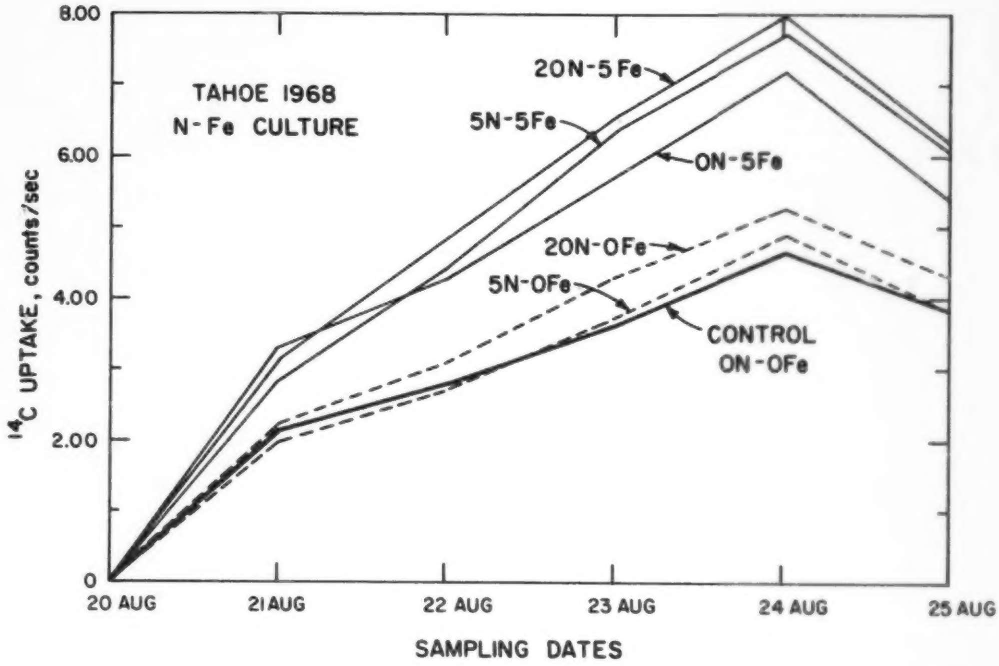

FIGURE 6. RESULTS OF NITROGEN AND IRON ADDITIONS TO LAKE TAHOE SURFACE WATERS

NOTE: Nitrogen was added as sodium nitrate and iron as ferric citrate. Note that both elements were somewhat stimulatory when added alone and when they were added together their stimulatory effects were additive.

DISCUSSION

The results of the Chlorella experiment provide a valuable comparison of the various methods currently being used for measuring the growth of algal cultures – cell count, optical density, and ^{14}C uptake. The cell count method for measuring growth was quite successful with the culture of Chlorella; the cell densities encountered were high enough so that statistically valid counts were obtained by direct counting with a haemocytometer. However, where fewer than 50 cells/mm^3 are found in a sample, the cells must be concentrated by centrifugation or filtration. Filters containing algae can be mounted and cleared on a microscope slide, giving a permanent record of the sample. This method is normally too slow and costly for laboratory cultures, but may be desirable for field work. Another way to count algal culture directly is by means of a 1-mℓ counting chamber utilizing a microscope with an ocular micrometer. Usually only a small portion of the chamber has to be counted to get valid counts, but a separate mount has to be made to identify the algal cells because the 1-mℓ chamber can only be viewed with low-power objectives. Algae in natural oligotrophic waters are often counted by means of Utermohl sedimentation chambers, a time-consuming and difficult method. Where different species are encountered in a sample, they have to be tabulated separately and measured so that some idea of the mass of the algae can be had.

The optical density method was the fastest and easiest method used in measuring the increase in the Chlorella culture. However, it is mainly applicable to measuring the concentration of dense cultures of algae. The most reasonable approach to solving problems connected with algal growth in natural waters is through the use of cell densities comparable to those found in the environment. At these densities population growth would be undetectable by the optical density method. Natural water commonly has a high ratio of detritus to living phytoplankton, so that only a small part of the optical density is due to living algae.

The growth of the culture was satisfactorily measured by the quick ^{14}C method. The results were expressed adequately by the counts per second measured on each filter. When experiments involve testing algal growth responses in water enriched with differing amounts of nutrients, this is best expressed as the percent increase of the counts over a control.

The C lorella experiment gave evidence that there was no detectable loss of ^{14}C from the cells during the fortuitous 24-hour dark period in the middle of the experiment, indicating that in this type of experiment the turnover rate of the ^{14}C is not great enough to obscure the results.

^{14}C data are best expressed as the rate at which carbon is assimilated at various stages of growth: note the Upper Truckee-Trout Creek and Reno STP effluent experiments (Figures 2 and 3, lower panels). Carbon assimilation data can only be calculated from experiments in which the ^{14}C is added to subcultures incubated for short periods of time. A subculture does not show the growth curve of the algae, but rather gives the first derivative with respect to time of the growth curve. In both sets of experiments above, the instantaneous growth rate reached a maximum and declined thereafter, although always remaining positive. The position of the maximum in instantaneous growth rate is itself an indication of the nutritive value of the medium. The maximum in the instantaneous growth rate curve signals the beginning of nutrient limitation in the culture. Of course this statement could only be valid for experiments that do not extend to the point where crowding or waste product levels become important controlling factors. In these experiments, the sampling interval was too long to accurately specify the point where the maximum was reached in each culture. It is only seen with certainty that the 10% and 50% additions of Reno secondary effluent sustained high growth rates longer than did the 1% additions of effluent or any of the Upper Truckee-Trout Creek additions.

The instantaneous growth rate can be used to measure accumulated growth simply by extrapolating the growth rate over the entire sampling interval. This is illustrated in Figures 2 and 3 in the middle panels showing the growth of Selenastrum in media prepared from filtered Lake Tahoe water with added portions of Upper Truckee-Trout Creek water and Reno STP effluent.

The 5-day bioassay of Upper Truckee-Trout Creek water using natural Lake Tahoe phytoplankton for test organisms illustrates the extreme sensitivity of the ^{14}C method relative to the cell count method (Table I and Figure 4). The cell count method does not give any indication that cell growth has occurred, yet the ^{14}C results show increased carbon uptake by the algae in the 10% culture compared to the other cultures. In all of the cultures the increasing radioactivities show that the algae are actively fixing inorganic carbon.

The bioassays of mineral nutrients using Tahoe surface water as a medium (Figures 5 and 6) illustrate further usages of the ^{14}C bioassay procedure. It is worth emphasizing that problems of calibration need not be considered when performing this type of experiment. Controls and experimentals are incubated together, all contain the same specific activity of ^{14}C, and all are made into membrane filter preparations of the same thickness for counting on the same Geiger-Mueller counter at the same efficiency. It is not even necessary to know the activity of the stock $Na_2{}^{14}CO_3$ solution accurately. No cell counts are available for the nitrogen and phosphorus experiment. Cell count data for the nitrogen and iron experiment revealed no significant differences between any of the cultures.

CONCLUSION

The ^{14}C method can easily be used for measuring algal cell growth in both nutrient-rich and nutrient-poor waters. ^{14}C experiments can detect growth in water with natural phytoplankton when cell count and optical density methods would not give evidence of any change in algal mass; ^{14}C data give a rate of carbon fixation, whereas cell count and optical density measurements are both static measures of mass. In addition, ^{14}C experiments can yield values proportional to the mass of cells in a culture.

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THE CLASSIFICATION OF LAKES

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INTRODUCTION

Man, in his quest to satisfy his curiosity concerning himself and his environment, is continually arranging things in an orderly fashion. He systematically probes at a problem until an understanding is developed which is usually soon entwined with a new terminology. He then begins to systematize his new findings with increasing sophistication as continued efforts with improved technology furthers his understanding. The intent of this orderly approach is to provide readily available information for use when a need is identified.

Aquatic ecosystems, and especially natural lakes, have not escaped this process. It all began with Forel (1892-1901) and his scheme of thermal classification. The subsequent contributors to lake typology literally make up a complete who's-who in limnology. Elster (1958) reviewed the history of lake typology and described the state of art to that time in the lead article to a section on lake classification, production, and consumption in Vol. 13 of the Proceedings of the International Association of Theoretical and Applied Limnology.

Since adequate reviews have been done on the research dealing with lake classification, and most of you are well aware of the classical works in this area, I will forgo the typical beginning of recapitulating the literature and summarize the bulk of the accomplishments toward lake systematics by saying that few if any of the physical, chemical, or biological features of lentic ecosystems have been left out of the numerous attempts at lake classification.

It was quite apparent from the onset that single environmental features (i.e., temperature, light, dissolved oxygen, pH, etc.) were not sufficient to meaningfully describe a lake. There were always exceptions which involved the interplay of some one or several other systems. The truly composite and integrated nature of aquatic ecosystems was manifesting itself. Indeed, each lake could be described in all its uniqueness to stand separate and different from any other. However, this serves no real purpose as the mass of data describing the individual systems becomes totally unmanageable. It is necessary to establish a system or systems by which we can orderly comprehend and manipulate the aquatic resources for whatever the intended use.

THE INTENDED USE

The question of the use to which such environmental arranging is to be put is paramount. The multiple use concept of water is well established as it applies to water in general; however, as it is applied to individual systems, it often is more conceptual than applicable. There is usually a prime use to which the other requests take positions of lesser importance. Before a meaningful scheme can be devised to type lakes, the objectives should be defined.

Uses can be grouped by the prepositions applied to each: use of, hydroelectric, agriculture, domestic, flood control; use on, boating, water skiing, swimming; use in, commercial and sport fishing; and use over, aesthetics. Though they are distinct in most cases, they are not altogether mutually exclusive. Thus, a classification scheme developed for one use may well suffice for several, although, a group of sport fishermen anchored in a productive bay may find it difficult to accommodate a legitimized water skiing use at the same place and time. It is not difficult to conjure up other equally conflicting water interests.

Classification schemes developed for specific water usages may not fully describe an aquatic ecosystem. It may be that a more comprehensive design would accomplish a better overall understanding of what lakes are really like. There is also the need to know the status of lakes in the evolutionary or succession process. Since change toward a terrestrial climax is taking place at some rate in each lake due to conditions without and within the lake, knowledge of the present situation can be most valuable in recording what the changes are and at what rate they are taking place. This is where those concerned with eutrophication become interested.

REGIONAL GROUPINGS

One of the first and most logical steps toward grouping lakes is on a regional basis. We seldom are interested from an applied sense in making comparisons among lakes of vastly different regions (e.g., arctic and tropics). The problem as always is what do you include in a region? What are the conditions that establish the boundaries? A geographical segregation into natural regions must consider geologic, edaphic, climatic, vegetational, etc. conditions. Margalef (1958) suggests that the requirements basic to a regional lake typology are: a) a relative constancy in the concentration of major mineral elements (Ca, Mg, Na, K, Sr, Cl, S); b) reckoning with differences arising from the respective histories and evolutionary processes and not only present ecological indicators; c) applicability of the scheme irrespective of numbers and/or size of the lakes in a region; d) allowance for the inclusion of different stages of succession in the same typology; e) a similarity in terrestrial conditions such as geologic substrate.

The classical work in regional lake typology in North America was that of the late D. S. Rawson (1960). The great quantity of information he collected on the lakes of Saskatchewan over several decades enabled him to make comparisons among lakes scattered over the granitic Precambrian Shield of the north and the southern glacial drift region (sedimentary). His primary purpose was to be able to better understand and manage the production of fish for which there was a substantial commercial fishery.

TROPHIC NOMENCLATURE

As a point of beginning, I will state without fear of too much challenge that the trophic nomenclature from oligotrophy to eutrophy is useful only in its broadest sense. It is possible to describe lakes at either end of the trophic spectrum, but it is not possible to discern the many subtleties existing in the extremities or the varieties of lakes spread along the continuum of the successional process. Though more elaborate nomenclatures have been developed, they function only as conversation pieces and are of little practical value since the distinctions between them are subjective and difficult to define. Such terms as hypolimnionistic eutrophy or 1-layer-ortho-oligotrophy are not household words even in limnological vernacular.

Since this symposium is concerned with eutrophication, the question to be asked now is how do you know where a particular lake is in the trophic successional process? What do you measure to give the needed information?

STATUS OF OREGON LAKES

For the past two years my students and I have been involved in a lake classification program for the lakes of Oregon. The overall objectives are first, to assemble all the available information on the lakes of the State into a central data bank; and second, to initiate a sampling program to give us the information needed to determine the present status of lakes where little or no information is available. The lakes to be sampled are selected on the basis of their accessibility to the public and the degree of their use. Four major regions have been designated: coastal, Cascade Mountains, high plateau (Eastern Oregon), and Wallowa Mountains. The coastal lakes have been receiving the bulk of the attention to date.

The program calls for at least two visits to each lake during the summer growing season and preferably at the time of maximum thermal stratification. A central sampling station is selected to represent the limnetic conditions. The following is a listing of the information collected for each lake.

I. Abiotic

  A. Physiography

    1. Location
    2. Origin
    3. Morphometrics
    4. Hydrology

  B. Physical

    1. Optical properties – light intensity and spectral composition with depth
    2. Thermal properties – temperature profiles and heat budgets

  C. Chemical

    1. Dissolved oxygen profiles
    2. pH profiles
    3. Alkalinity profiles
    4. Redox potential profiles
    5. Total hardness profiles
    6. Specific conductance
    7. Total dissolved solids

II. Biotic

A. Limnetic phytoplankton

1. Qualitative — species inventory
2. Quantitative
    a. Pigment analysis
    b. Photosynthetic rate — in situ carbon-14

B. Zooplankton

1. Littoral — qualitative only
2. Limnetic — replicate vertical tows with metered standard tow net (#12 mesh)

C. Macrophytes

1. Qualitative — submergent, emergents and beach associations
2. Quantitative — ratio of the area of littoral vegetation to open water area

The data are punched onto cards and stored for eventual computer processing. We are in the process of adapting the techniques of "numerical taxonomy" to grouping our lake data for meaningful comparisons. This is the approach being used by A. L. Sheldon in his lake classification work with Resources for the Future, Inc. Multivariate statistical methods such as discriminant function analysis are being considered for comparative analysis. The problem of developing a definition between similarity and dissimilarity is still unsolved. It is not difficult when lake groups are fairly well defined and the differences are rather obvious. When the differences are less distinct, the problem magnifies.

WHAT IS OLIGOTROPHY?

Within our general lake classification effort we are conducting more extensive, as well as intensive, sampling of four lakes which have heretofore all been labeled oligotrophic. I would like to share some of these preliminary data with you in order to demonstrate the problem extant in selecting appropriate classification criteria.

The lakes differ in location, origin, and basic morphometrics as seen in Figures 1 and 2 and Table I. The differences in basic chemistry (Table II) are rather significant for supposedly oligotrophic ecosystems. Crater Lake certainly appears to be better off in regard to its basic chemistry than the other three. The thermal and dissolved oxygen profiles (Figure 3) all indicate classical oligotrophic conditions for all four lakes.

When we compare the photosynthetic rates for a midsummer sampling period (July 1968), the picture changes (Figures 3 and 4). There is one order of magnitude difference in the carbon assimilation scale from Crater Lake to Woahink Lake and another full order from Woahink Lake to Odell Lake. Primary productivity measurements will begin in Waldo Lake this coming summer.

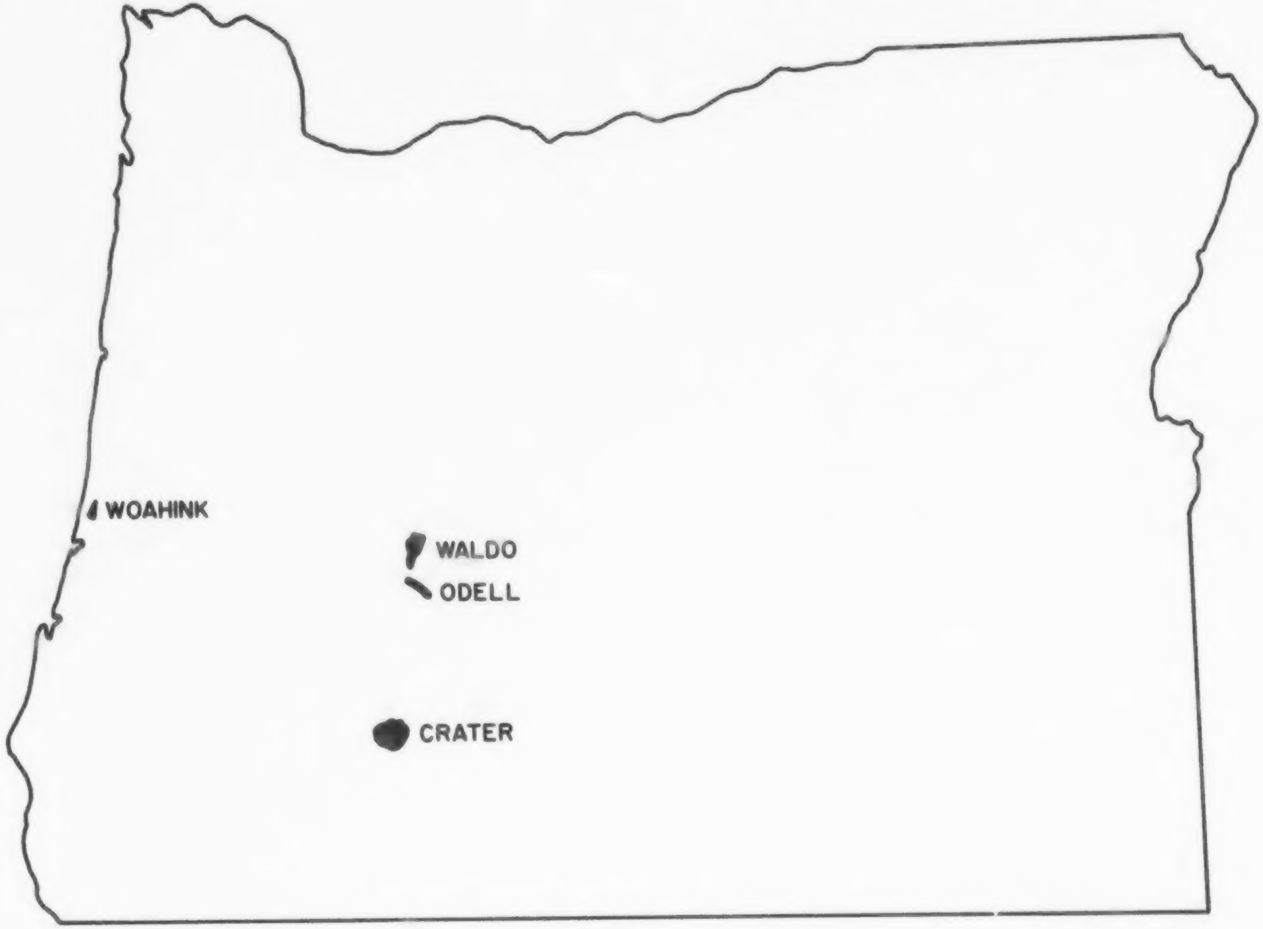
WOAHINK
WALDO
ODELL
CRATER

FIGURE I. MAP OF THE STATE OF OREGON SHOWING LOCATION OF THE FOUR OLIGOTROPHIC STUDY LAKES

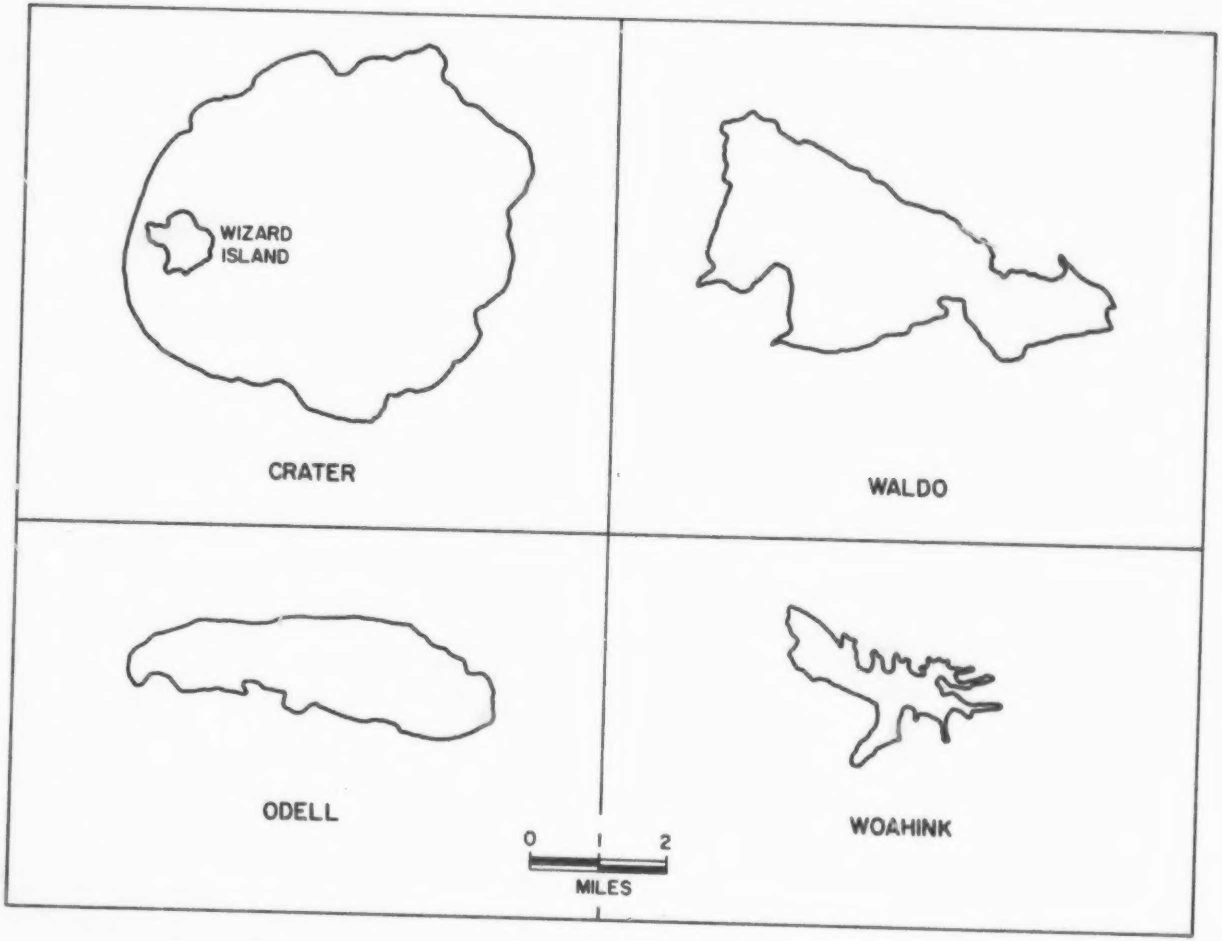
WIZARD ISLAND
CRATER
WALDO
ODELL
WOAHINK
0
1
2
MILES

FIGURE 2. OUTLINE MAPS OF OLIGOTROPHIC STUDY LAKES IN OREGON SHOWING COMPARATIVE SHAPES AND SIZE

TABLE I

BASIC MORPHOMETRIC DATA FOR FOUR OLIGOTROPHIC LAKES IN OREGON

	Crater	Waldo	Odell	Woahink
Location	Cascade Range	Cascade Range	Cascade Range	Oregon Coast
Elevation	6200 ft	5400 ft	4800 ft	Sea Level
Origin	Volcanic Caldera Basin	Glacial Scar Enclosed By 300 ft Thick Lava Dam	Glacial Trough Enclosed By Intersecting Lava Flow	Sand Dune Encroachment Of River Valley
Area	5260 ha	2600 ha	1420 ha	325 ha
Z_M	589 m	128 m	88 m	21 m

TABLE II

COMPARATIVE CHEMICAL FEATURES OF FOUR OLIGOTROPHIC LAKES IN OREGON

	Crater	Waldo	Odell	Woahink
Total Alkalinity (mg/ℓ $CaCO_3$)	28.0	1.6	16.5	8.2
Total Hardness (mg/ℓ $CaCO_3$)	27.4	0.9	10.6	8.6
Total Dissolved Solids	81.5	25.5	26.9	44.7

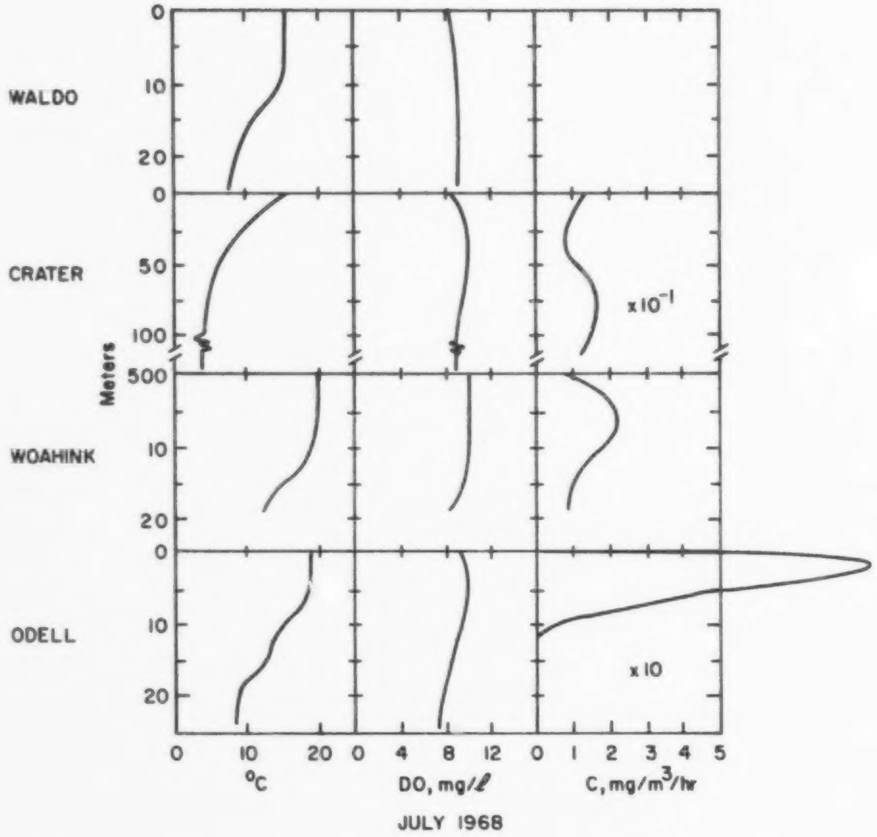

FIGURE 3. THERMAL, DISSOLVED OXYGEN AND PRIMARY PRODUCTION (*in situ*, FOUR-HOUR ^{14}C INCUBATION) DEPTH PROFILES FOR FOUR OLIGOTROPHIC OREGON LAKES

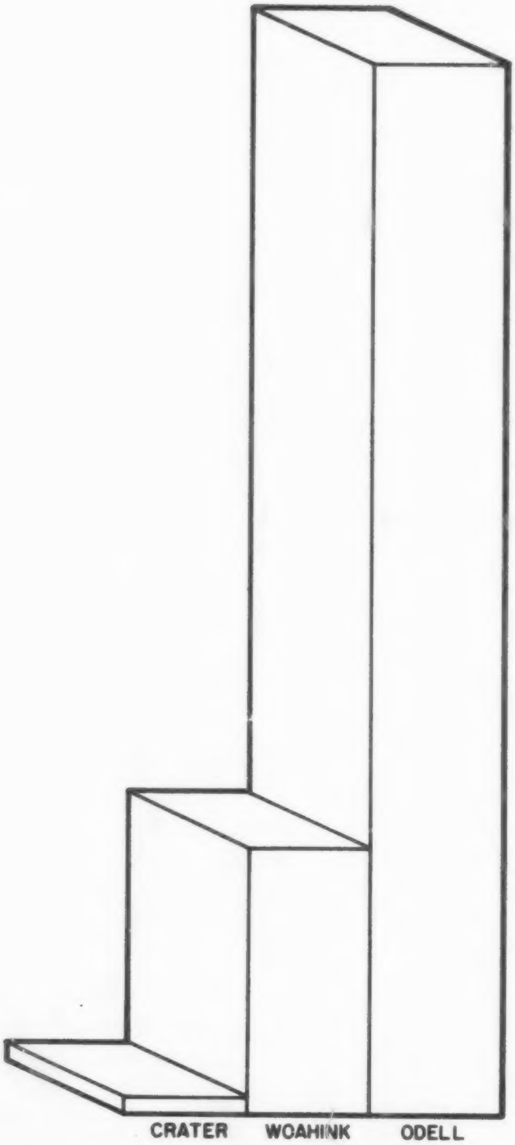

FIGURE 4. COMPARATIVE TOTAL PHYTOPLANKTON PRODUCTION IN mg ^{12}C FOR JUNE THROUGH AUGUST, 1968 IN THREE OLIGOTROPHIC OREGON LAKES

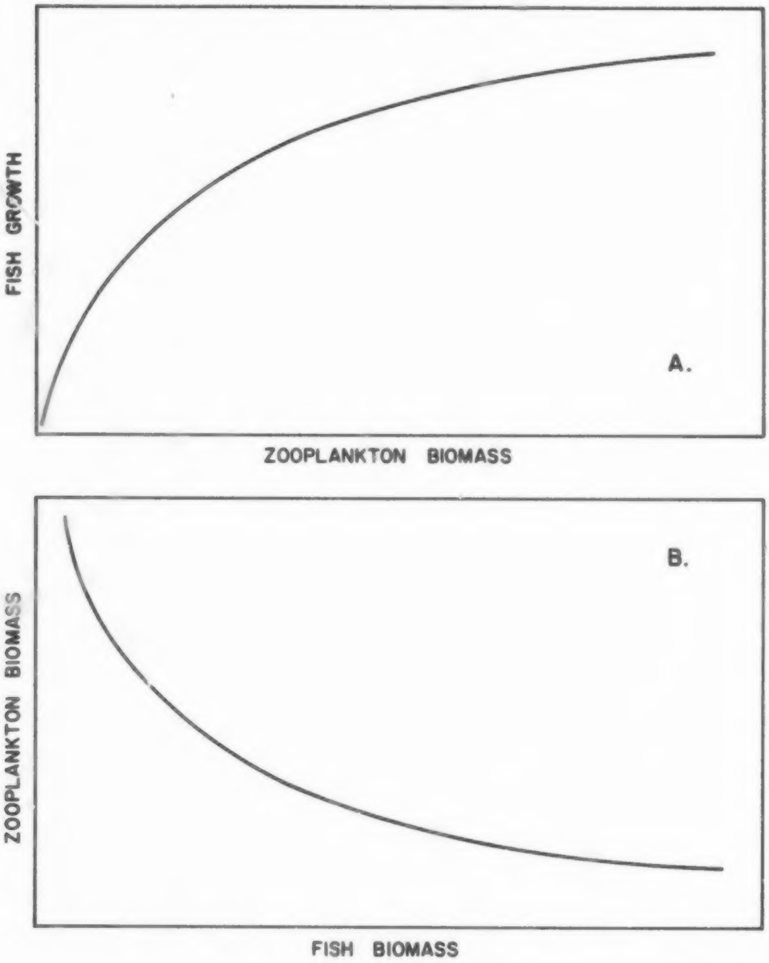

FIGURE 5. THEORETICAL CURVES, A. AN ECOSYSTEM WHERE FOOD IS EVENTUALLY LIMITING, INDICATING THE RELATIONSHIP BETWEEN GROWTH RATE OF A FISH SPECIES AND THE DENSITY OF ITS FOOD; B. BETWEEN THE DENSITY OF A PREY ORGANISM AND THE DENSITY OF ITS PREDATOR. (ADAPTED FROM BROCKSEN, DAVIS, AND WARREN, 1969)

Using only physical and chemical data for comparing the trophy of three of these four lakes would have been quite misleading. In order to better understand the true trophy of a lake it is essential to have some measure of its biotics. Photosynthetic organisms and particularly limnetic phytoplankton represent the only trophic level that can be practically isolated without regard to species. Using convenient and sensitive techniques for measuring photosynthetic rates, it is possible to establish production values for phytoplankton. Such convenient techniques, however, are not available for animal populations and we must resort to biomass estimates and involved growth-rate measurements to arrive at production. Such measurements can be made only on individual species and not communities or trophic levels. Because of the degree of difficulty in the measurements, such information is nearly totally wanting for natural populations of consumers. Thus, it is apparent that Lindeman's (1942) trophic dynamic approach to an ecosystem is theoretically sound but is nearly impossible to quantify beyond the primary procedures.

DENSITY-DEPENDENCE CONSIDERATIONS

I would now like to borrow rather heavily from the recent and very exciting work of three of my colleagues at Oregon State University in an attempt to develop a sounder biological basis for lake classification. Brocksen, Davis, and Warren (1968), writing on the production of sculpins and trout in laboratory streams, first proposed that the density of a prey organism in relation to the biomass of its predator may be the logical approach to understanding trophic dynamics. In a second paper (Brocksen, Davis, and Warren 1969), they further refine their analysis of the trophic process on the basis of density-dependent functions.

They point out that by considering only broad and ill defined trophic levels, we ignore the individual species and their specific food needs. They state "... it is hard to conceptualize how species compete for the production rate of a food resource rather than for existing biomasses of food." Furthermore, there is little evidence to date that ecologists have been successful in relating predator production to the production rates of even the more important prey species.

These same authors propose that it is "... prey density and distribution rather than production rate that immediately determine the amount of food a predator can obtain and the energetic costs of its pursuit." Density of a food resource is the resultant of the total output through production and the outflow through consumption and decomposition; thus, it becomes a real measure of the outcome of changes in these rates or of environmental effects upon them. Prey biomass, in addition to being perhaps the best measure of prey availability, is simpler to measure than prey production.

Brocksen, Davis, and Warren (1969) present considerable evidence showing that growth rates of consumers in nature are often simple functions of densities or biomasses of their food organisms and that the densities of prey organisms are often simple functions of predator densities. These relationships apply to ecosystems where food is a limiting resource. In Figure 5 I have condensed their ample graphical evidence of the relationships between food density and consumer growth rate and between consumer density and food density into hypothetical curves applying to zooplankton and young sockeye salmon feeding in a lake.

What happens when food remains the limiting resource and you compare these same relationships among different ecosystems with different capacities for food production? Brocksen, Davis, and Warren (1969) used data from three sockeye salmon-producing lakes to show these relationships. I have drawn the curves in Figure 6 to represent hypothetical conditions of the biomasses of phytoplankton and zooplankton in a series of lakes where food is limiting. The same relations would be expected for zooplankton and young sockeye salmon. The assumption is that lakes having the highest basic capacity to produce food should maintain the highest biomasses of a consumer. Thus, the relationship among lakes is positive. The negative slope of the line for each lake will be maintained as long as the basic productive capacity is constant. The decreases in negative slope from ecosystem to ecosystem as productivity increases are intended to show that in ecosystems of higher productive capacity the consumption of phytoplankton by zooplankton becomes a less important fate.

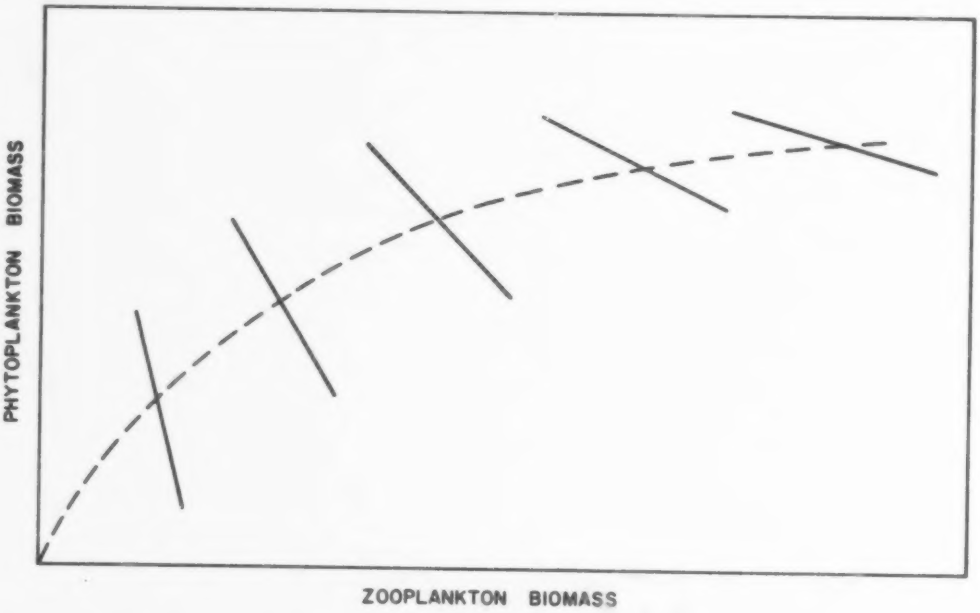

FIGURE 6. THEORETICAL RELATIONSHIP OF PREY-PREDATOR BIOMASSES (SOLID LINES) WITHIN LAKES OF VARYING PRODUCTIVITY AND BETWEEN THESE SAME LAKES (DASHED LINE). (TAKEN FROM BROCKSEN, DAVIS, AND WARREN, 1969).

The same argument can be applied to nutrients and the biomass, growth and production of phytoplankton. Again for any ecosystem having a given resource of plant nutrients, an increase in phytoplankton density is accompanied by a decrease in nutrient concentration. The curves in Figure 5 can also be used to show the relationship between both phytoplankton growth-nutrient concentration and phytoplankton biomass-nutrient concentration. For ecosystems of differing nutrient concentration the relationship between nutrients and phytoplankton biomass should also resemble Figure 6.

Since the food available to an organism in a food-limiting environment diminishes as its biomass increases, it follows that individual growth rates in the consumer population will decrease. Production being the product of growth rate and biomass, we thus have established a relationship between biomass and production. These relationships are presented for three hypothetical lakes of differing productivity in Figure 7. Mathematical models based on these relationships allow one to estimate the biomass that would maximize production; it is possible to predict what kinds of changes in the consumer, its food organisms, or its competitors will alter the biomass-production relationship. In order to arrive at production of the organism of interest, it is necessary to know both biomass and growth rate. The estimate of growth rate is by far the more difficult to accomplish. Except for fish populations, seldom are such data available.

Brocksen, Davis, and Warren (1969) developed a production model based on what they call the dynamic-biomass equilibria. Once the relationships we have been considering are mathematically defined, it is possible to model the production of a consumer of interest as functions of its biomass, the biomass of its competitors, and the density of their prey. Such a model would take into account the important interactions of predators, competitors, and prey and thus provide a more realistic estimate of the biomass of consumer that would maximize its production. I have purposefully omitted the modeling developed by these authors because of time and space limitations and suggest you refer to the original papers if you are interested.

Whenever their relationships can be defined, there is a sound logical basis for their use in developing such models. There seems to be ample support for the use of density-dependent relationships in establishing production estimates for species of interest in an aquatic ecosystem. By defining relationships between nutrients and primary producers, production models can be developed that allow prediction of the effect of nutrient changes on consumer biomass. This has great significance in not only better understanding the trophic process, but providing useful information for the actual management of aquatic resources.

Knowing the density-dependent relationships existing within a single lake, as well as among lakes, could greatly aid in establishing a meaningful lake classification scheme. Of greater significance is the possibility of being able to plot the trophic evolution of a lake through the biota instead of just physically and chemically.

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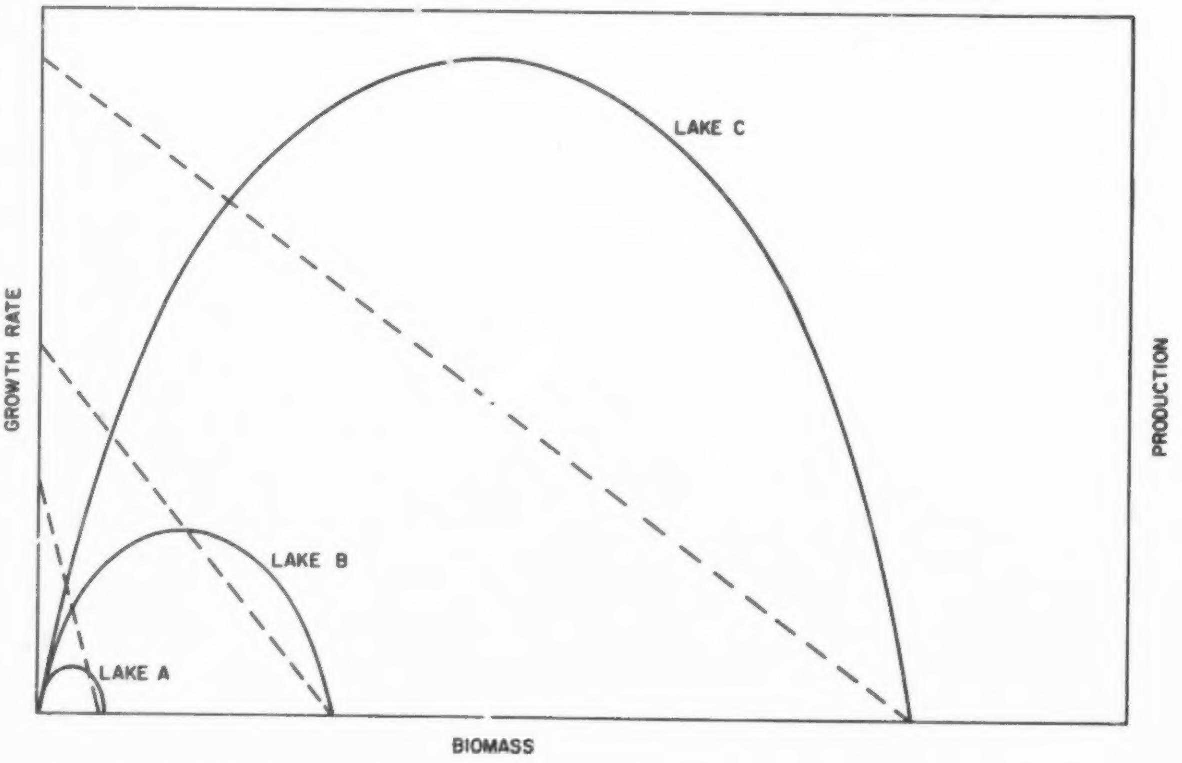

FIGURE 7 THEORETICAL RELATIONSHIPS BETWEEN GROWTH RATE AND PRODUCTION OF THE BIOMASS OF ANY PARTICULAR LIMNETIC ORGANISM IN THREE HYPOTHETICAL LAKES OF VARYING PRODUCTIVITY

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PRODUCTIVITY OF RIVERS

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INTRODUCTION

A considerable stimulation of interest in the productivity of rivers has resulted since the publication of Odum's (1956) paper on primary production of flowing waters. In the paper, Odum suggested the use of the upstream-downstream measurement of oxygen, carbon dioxide, and other properties for the study of flowing water communities. From the observed daily curves of oxygen or carbon dioxide in streams, the component rates of photosynthesis, respiration, and diffusion can be calculated. Odum also recommended that the ratio of gross photosynthesis to total community respiration be used to classify communities quantitatively according to their predominantly heterotrophic or autotrophic characteristics. He postulated that longitudinal succession within a stream tends to modify the ratio toward unity from higher values for autotrophic and from lower values for heterotrophic communities.

In subsequent papers, Odum (1957 a, b) reported on the results of productivity measurements of springs. Using oxygen changes Hoskin (1959), Edwards and Owens (1962), Copeland and Duffer (1964), and Duffer and Dorris (1966) estimated production in rivers. McConnell and Sigler (1959) were unable to use the oxygen balance method in the Logan River because turbulence resulted in equilibration of the water with the atmosphere at all times.

The author's interest in studying the productivity of the Madison, Firehole, and Gibbon rivers was aroused by Armitage's (1958) study of the ecology of riffle insects of the Firehole River, Wyoming. The Firehole River (Figure 1) arises in Madison Lake, Yellowstone National Park, and flows in an essentially northerly direction until its junction with the Gibbon at Madison Junction. For about twelve miles it flows successively through the Upper, Midway, and Lower Geyser basins. The discharge of thermal waters entering the Firehole has been estimated by Allen and Day (1935) to be 54.87 cfs (1.57 m^3/sec). Of this total discharge, 18.70 cfs (0.55 m^3/sec) is contributed by the Upper Geyser Basin, 12.94 cfs (0.36 m^3/sec) by the Midway Geyser Basin, and 23.23 cfs (0.66 m^3/sec) by the Lower Geyser Basin. The Gibbon River receives a much smaller discharge of thermal water (6.19 ft/sec or 0.19 m^3/sec). As a result of hot spring discharge there is a progressive downstream increase in dissolved solids and an elevation in temperature (Table I). Armitage (1958) demonstrated that the average yearly standing

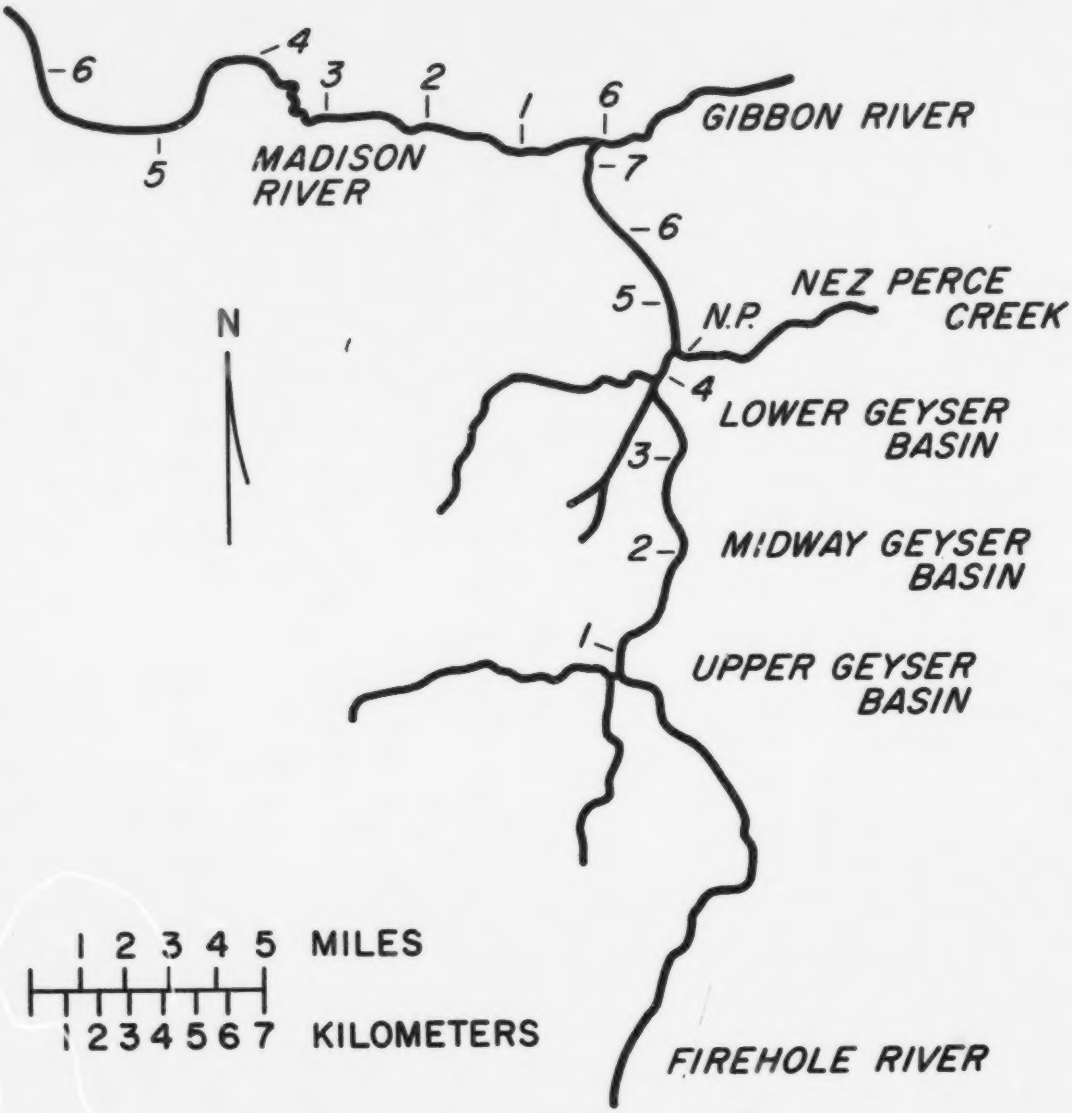
6
5
4
3
2
1
6
GIBBON RIVER
MADISON RIVER
7
6
5
N.P.
NEZ PERCE CREEK
4
LOWER GEYSER BASIN
3
2
MIDWAY GEYSER BASIN
1
UPPER GEYSER BASIN
N
1 2 3 4 5 MILES
1 2 3 4 5 6 7 KILOMETERS
FIREHOLE RIVER

FIGURE I. LOCATION OF SAMPLING STATIONS

TABLE I

CHEMICAL ANALYSIS OF RIVERS AFFECTED BY THERMAL DRAINAGE

(me/ℓ)

River	$\kappa_{25} \times 10^6$ Conductivity	Ca	Mg	Na	K	HCO_3	Cl	SO_4	PO_4	SiO_2
Upper Gardner	231	1.15	0.63	0.43	0.06	1.83	0.22	0.56	0.003	1.14
Lower Gardner	493	2.31	1.48	1.09	0.35	2.61	0.74	2.52	0.008	1.18
Upper Gibbon	108	0.20	0.06	0.54	0.10	0.62	0.14	0.54	0.004	1.99
Middle Gibbon	283	0.22	0.09	2.15	0.22	1.11	1.16	0.80	0.007	2.21
Lower Gibbon	353	0.23	0.09	2.95	0.25	1.71	1.22	0.77	0.009	2.27
Upper Firehole	92	0.16	0.07	0.59	0.08	0.39	0.41	0.10	0.008	1.61
Middle Firehole	423	0.18	0.03	4.30	0.21	1.95	1.73	0.16	0.017	2.53
Lower Firehole	459	0.17	0.05	4.19	0.21	2.46	1.96	0.38	0.018	2.49
Upper Madison	429	0.20	0.09	3.61	0.25	2.04	1.69	0.46	0.013	2.39
Middle Madison	424	0.14	0.04	3.82	0.28	2.13	1.58	0.44	0.013	2.42
Lower Madison	404	0.26	0.03	3.68	0.22	1.96	1.52	0.40	0.015	2.33

crop of Firehole River and other Yellowstone Park streams was positively correlated with temperature and bicarbonate alkalinity, the latter being more significant. Furthermore, the relative abundance of insect orders also changed with change in average alkalinity. Trichoptera was positively correlated with alkalinity and Ephemeroptera was negatively correlated. Vincent (1967) found the same situation to occur in the Gibbon River.

It seemed apparent that the Firehole, Gibbon, Madison River system offered an ideal situation to study the effects of natural thermal and chemical enrichment on flowing water communities and their productivity.*

Seven sampling stations were established on the Firehole River and six on the Madison River, as well as a station at the mouths of the Gibbon River and Nez Perce Creek. (See Figure 1.) Six series of samples at weekly intervals were taken from July 22 to September 1, 1964 from the Madison River stations and ten series were obtained from all stations at weekly intervals from July 14 to September 15, 1965.

As reported by Wright and Mills (1967), oxygen could not be used as an index of photosynthesis as the river was equilibrated with atmospheric oxygen at all times. However, diurnal pH curves were quite distinctive (Figure 2) and indicated that the relationship between pH and total CO_2 could be used to measure photosynthesis. Titration curves relating pH and total CO_2 were obtained by titrating water samples with carbon dioxide-saturated water as described by Beyers and Odum (1959).

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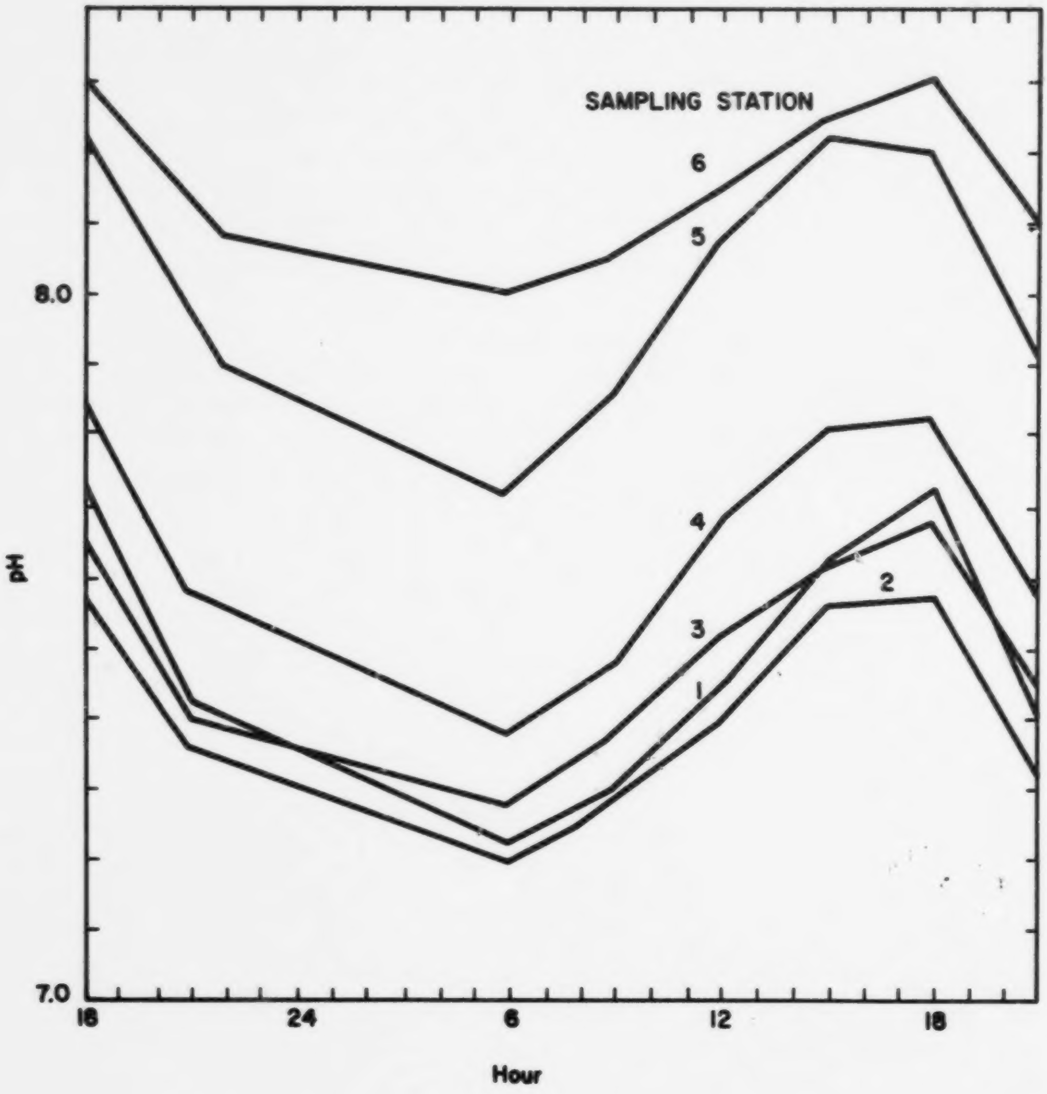

FIGURE 2. DIURNAL pH CHANGE, MADISON RIVER, AUGUST 10-11, 1964

MADISON RIVER STUDIES

Net photosynthesis during the day and respiration during the night were computed by the upstream-downstream method (Odum 1956) as follows:

$$P - R = \frac{h\,(C_1 - C_2)}{t} - k\,(C_w - C_a) - A_c$$

where P = uptake of CO_2 by photosynthesis ($g/m^2/min$)

R = gain of CO_2 by respiration ($g/m^2/min$)

C_1 = downstream CO_2 concentration (g/m^3)

C_2 = downstream CO_2 concentration (g/m^3)

t = flow time between stations (min)

k = evasion coefficient of CO_2 in water (m/min)

C_w = concentration of CO_2 in water (g/m^3)

C_a = concentration of CO_2 in atmosphere (g/m^3)

A_c = Accrual rate of CO_2 ($g/m^2/min$)

Average values of net and gross photosynthesis and respiration from the six series of 1964 samples are shown in Table II and Figure 3. With the exception of reach 3, the rates of gross and net photosynthesis and respiration declined downstream. The downstream decline in metabolism was paralleled by a decline in standing crops of macrophytes. It is apparent that photosynthesis becomes progressively limited downstream.

TABLE II

AVERAGE VALUES OF NET AND GROSS PHOTOSYNTHESIS AND RESPIRATION ($g\ C/m^2/day$)

	Reach					
	1	2	3	4	5	Avg.
Net photosynthesis	1. 7	1. 1	2. 0	0. 9	0. 3	1. 2
Respiration	-1. 7	-1. 0	-0. 4	+0. 2	-0. 1	-0. 6
Gross photosynthesis	3. 4	2. 1	2. 4	0. 7	0. 4	1. 8

Phosphate phosphorus concentrations were much higher than those in highly eutrophic waters (0. 28-0. 41 ppm). However, nitrate-nitrogen concentrations as determined by the method of Mullin and Riley (1955) were always less than 1 ppb. Ammonia nitrogen as determined by the method of Richard and Kletsch (1964) averaged 13 ppm and there was no significant difference in concentration between stations.

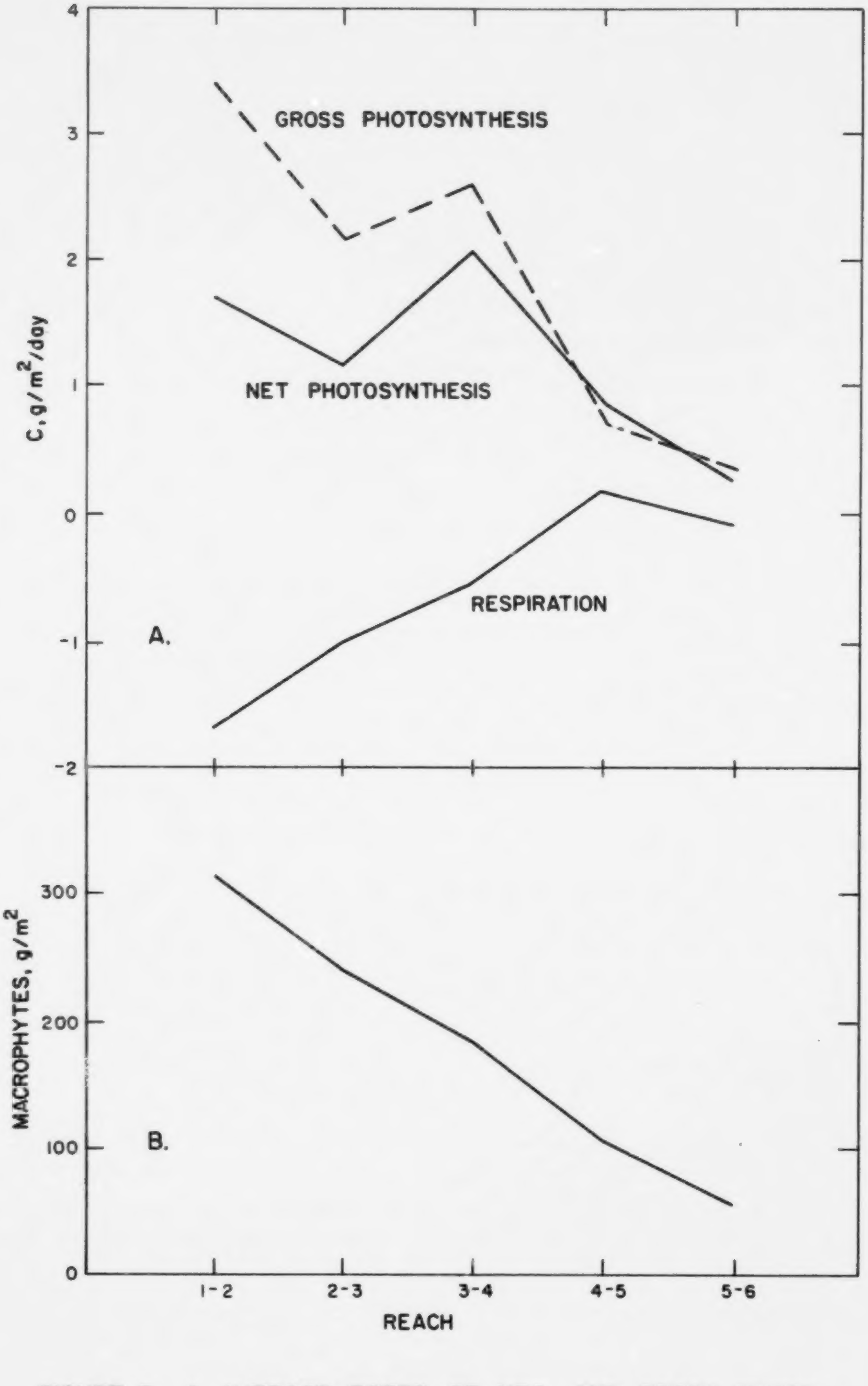

FIGURE 3. A. AVERAGE RATES OF NET AND GROSS PHOTOSYNTHESIS AND RESPIRATION IN THE MADISON RIVER; B. AVERAGE STANDING CROP OF MACROPHYTES

High photosynthetic rates were correlated with high light intensity in the upper three reaches (Figure 4), although there is considerable scatter in the points. In the lower two reaches, increasing light intensity had relatively little effect on photosynthesis.

Obviously something besides light phosphorus and nitrogen was limiting productivity in the lower reaches. The only substance that decreased downstream was free CO_2. When free CO_2 concentrations were plotted against photosynthetic rates (Figure 5), it became obvious that at low concentrations of free CO_2 the rate of photosynthesis was depressed; as free CO_2 increased the rate of net photosynthesis increased in a nonlinear fashion.

To illustrate the interaction between light and free CO_2 concentration, the value of photosynthesis corresponding to particular light intensities and free CO_2 concentrations were plotted and a series of curves was drawn to illustrate the minimal values necessary for attainment of a particular rate of photosynthesis (Figure 6). Only at high light intensities and high free CO_2 concentrations were the maximal rates observed. With constant CO_2 concentration and a decrease in light intensity, photosynthesis decreased. Conversely, with constant light intensity and a decrease in free CO_2, photosynthesis also decreased.

The greater amounts of plant growth and higher rates of photosynthesis in the upper reaches are correlated with high concentrations of free CO_2. Photosynthesis would seem to be more light-limited in this part of the river than CO_2-limited. The lesser amounts of plant growth and decreased photosynthetic rates in the lower reaches are correlated with low concentrations of CO_2. The river here tends to be more CO_2-limited than light-limited.

COMPARATIVE STUDIES OF THE FIREHOLE, GIBBON, MADISON RIVERS, AND NEZ PERCE CREEK

Averages of the 1965 results are presented in Figure 7. Firehole stations 1 and 2 were located below the Upper Geyser Basin and above the Midway Geyser Basin. Average total alkalinity was 55 and 62 ppm CO_3 and the rate of photosynthesis was 0.53 g C/m^3/day. Firehole station 3 was located above the Lower Basin and Firehole station 4, below. Total alkalinity averaged slightly above 100 ppm and the photosynthetic rates exceeded 1.0 g C/m^3/day. Nez Perce Creek with a total alkalinity of 72 ppm had an average photosynthetic rate of 0.45 g C/m^3/day. Firehole stations 5 and 6, which were located below the confluence of Nez Perce Creek and the Firehole River, had average total alkalinities of approximately 100 ppm and photosynthetic rates between 0.9 and 1.0 g C/m^3/day. Firehole station 7, even though it had an average total alkalinity of 95 ppm, exhibited the lowest photosynthetic rate — 0.53 g C/m^3/day. The total alkalinity of the Madison River stations was approximately 100 ppm. Photosynthesis of the four upstream stations was high (0.92-1.04 g C/m^3/day). However, Madison River stations 5 and 6 were characterized by decreased rates of photosynthesis (0.82 and 0.59 g C/m^3/day).

The data indicate that the highest photosynthetic rates occurred only when total alkalinity was high (approximately 100 ppm). On the other hand, Firehole station 7 and Madison River stations 5 and 6 were characterized by lower rates even though the total alkalinities were $\pm$100 ppm. As previously mentioned, photosynthesis was apparently limited by low free CO_2 concentrations at these stations.

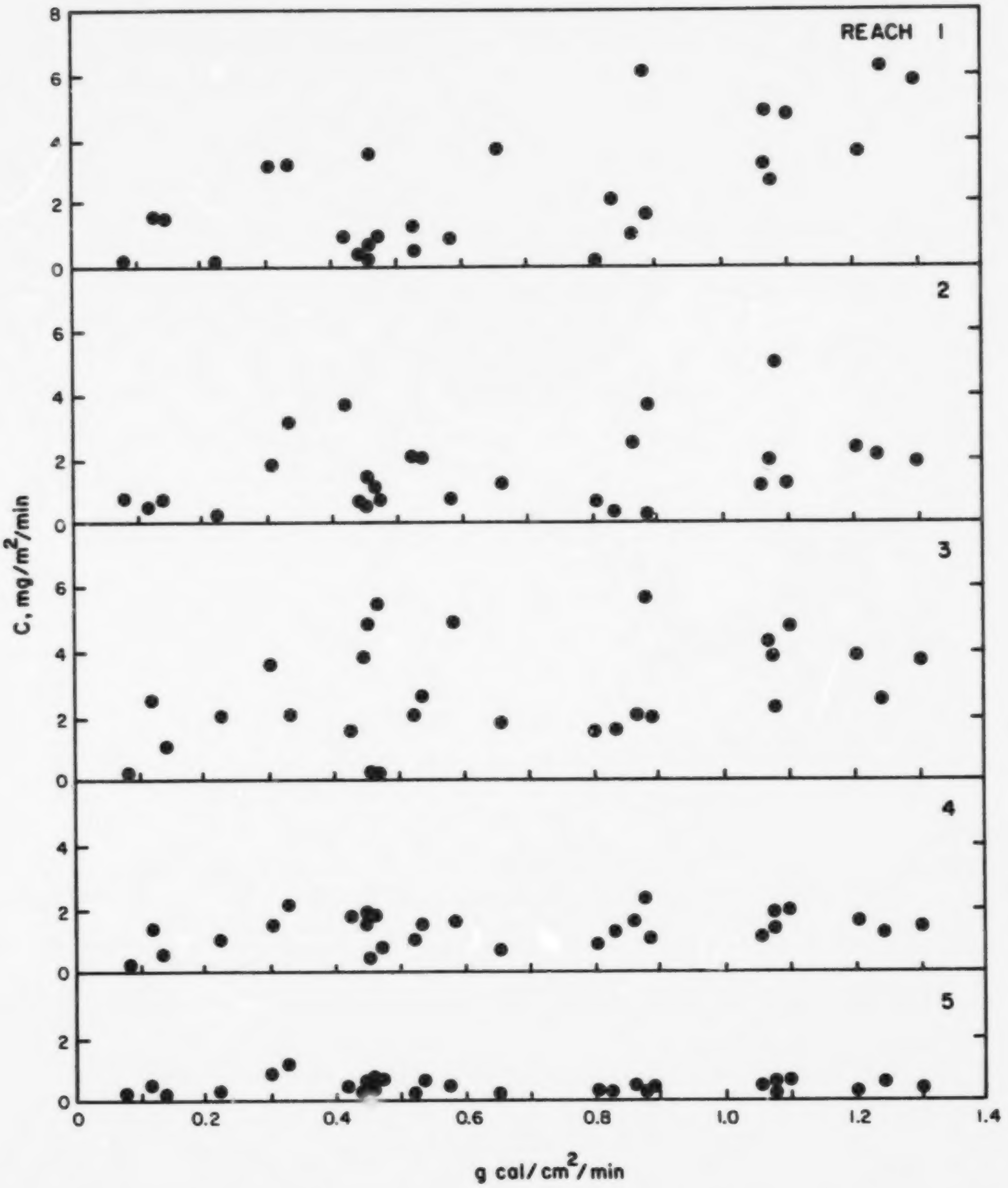

FIGURE 4. NET PHOTOSYNTHETIC RATES THAT OCCURRED DURING 3-HR SAMPLING PERIODS PLOTTED AGAINST SOLAR RADIATION

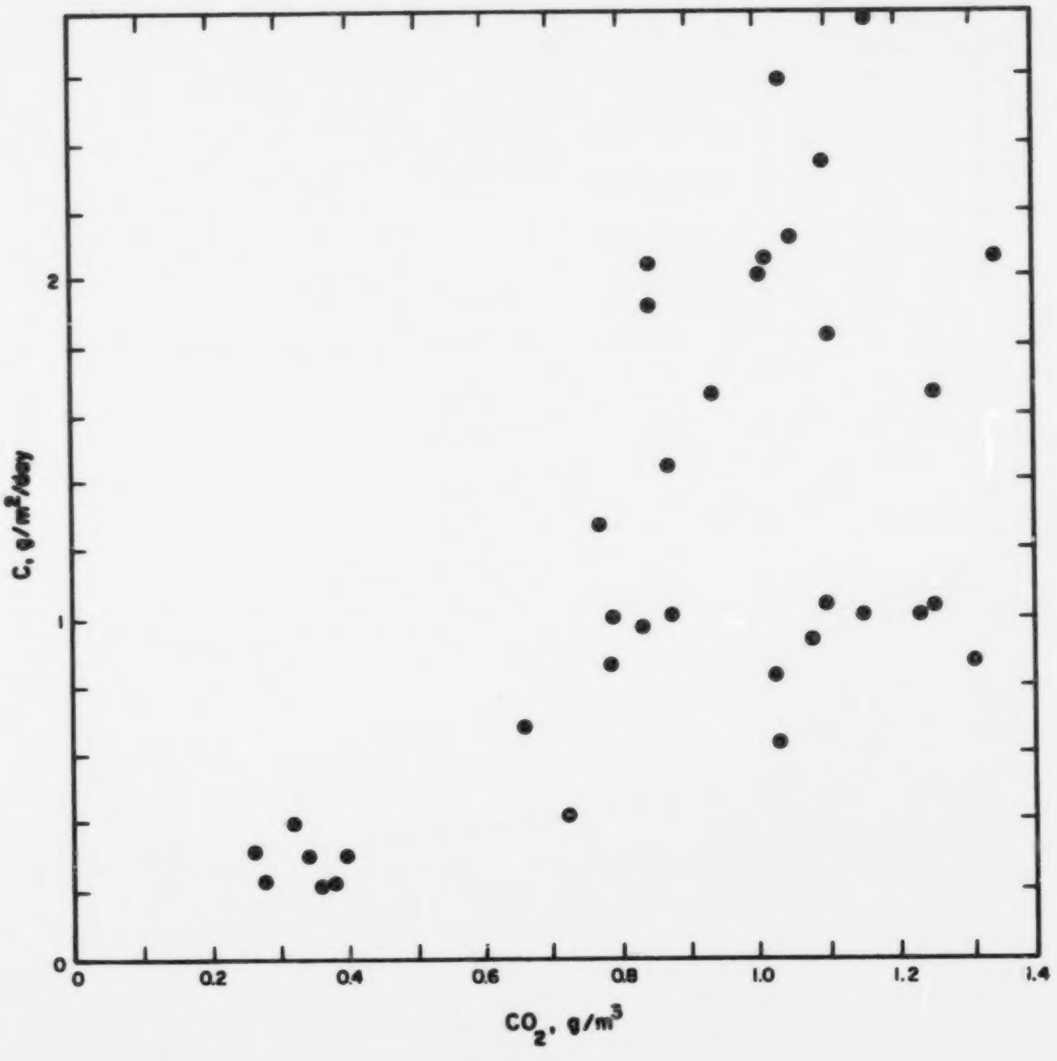

FIGURE 5. DAILY NET PHOTOSYNTHESIS PLOTTED AGAINST THE FREE CO_2 CONCENTRATION, 0600 HOURS

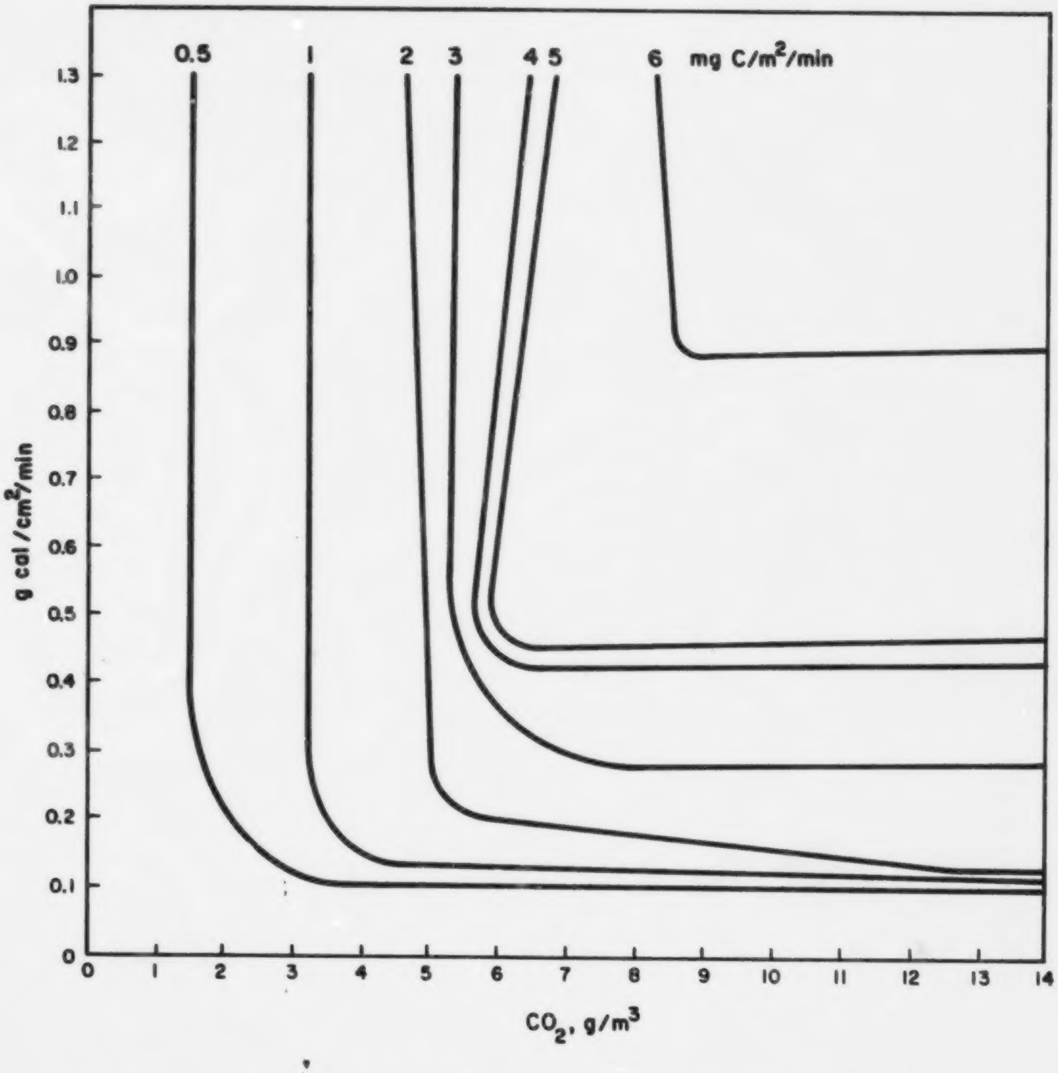

FIGURE 6. ISOCLINES OF PHOTOSYNTHETIC RATES REPRESENTING THE LIGHT INTENSITY AND CO_2 CONCENTRATION NECESSARY FOR A PARTICULAR PHOTOSYNTHETIC RATE DURING A 3-HR PERIOD

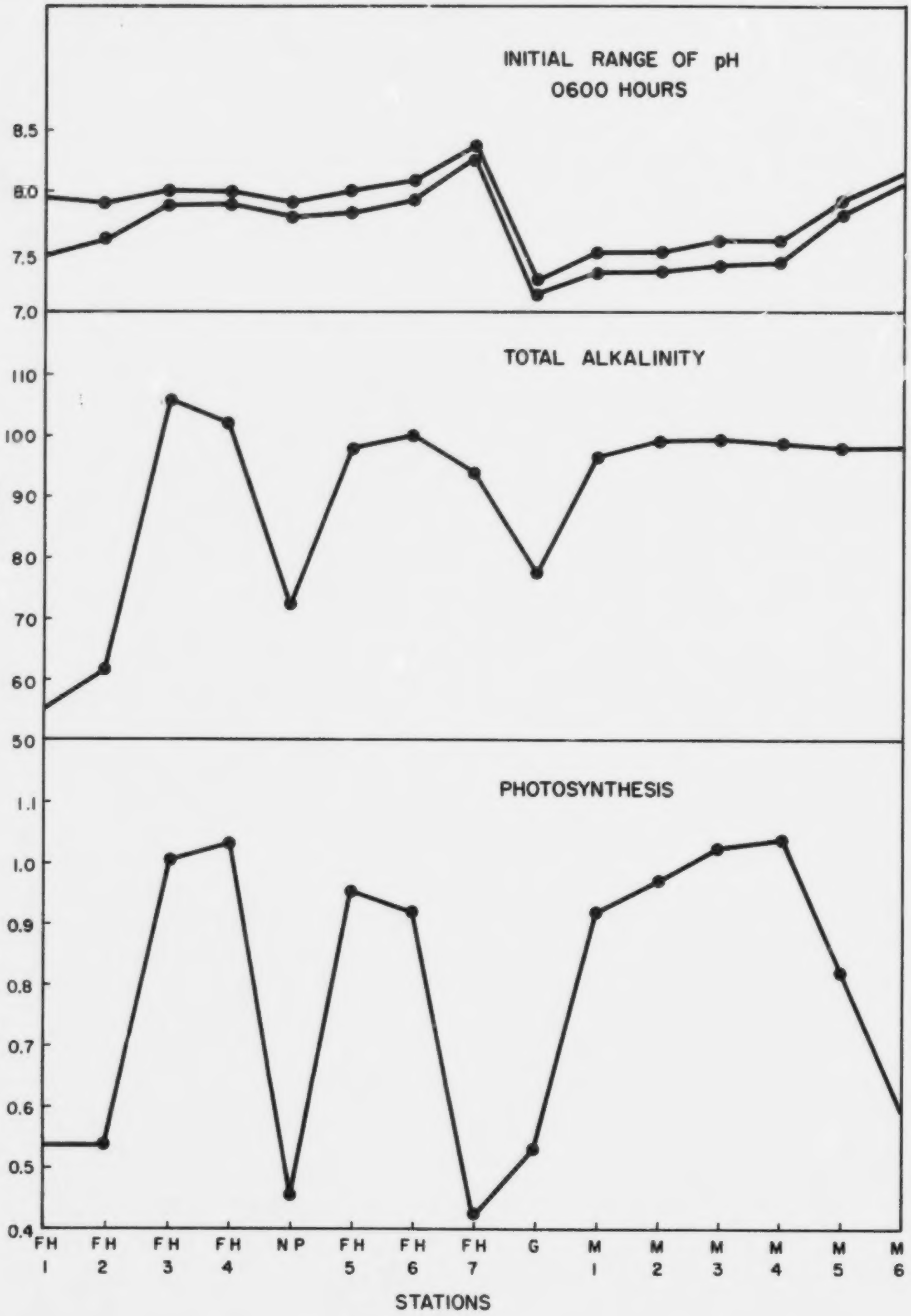

FIGURE 7. COMPARISON OF AVERAGE NET PHOTOSYNTHESIS AT THE FIREHOLE, GIBBON, MADISON RIVERS AND NEZ PERCE CREEK WITH CORRESPONDING VALUES OF AVERAGE TOTAL ALKALINITY AND RANGE OF pH VALUES

SOLUBLE ORGANIC CARBON AND NITROGEN IN RELATIONSHIP TO PHOTOSYNTHESIS

In 1966, a series of 12 weekly determinations were made of soluble, organic carbon, soluble organic nitrogen, and ammonia in relationship to photosynthesis. The samples were collected at each of the six Madison River stations at three-hour intervals between 0600 and 2400 hours and at a six-hour interval from 2400 to 0600 hours. The average results of all determinations are portrayed in Figure 8.

Photosynthetic uptake of inorganic carbon amounted to 1.22 g C/ℓ between 0600 and 1500 hours. Between these hours there was an increase of 0.25 f/ℓ of soluble organic carbon. The increase of soluble organic carbon amounted to 20% of the photosynthetic uptake. Concurrently with the increase of soluble organic carbon there was a two fold increase in organic nitrogen. The carbon-nitrogen ratio decreased during the same interval.

After 1500 hours respiration became dominant over photosynthesis and there was a decline in soluble organic carbon and nitrogen accompanied by an increase in the carbon-nitrogen ratio.

The curve for ammonia-nitrogen is not what one would expect. There was a steady increase of ammonia-nitrogen from 0600 to 2400 hours followed by a decline. Ammonia nitrogen was the only measurable form of nitrogen available to the aquatic flora and one would have expected that the concentration would have declined parallel to the curve for photosynthetic uptake of carbon. Either the rate of uptake of ammonia was less than the rate of ammonification or nitrogen fixation was occurring.

It appears without question that the increase of dissolved organic matter during the period of active photosynthesis represents organic compounds that were synthesized and which, subsequently, were released by the plants.

Acting under the assumption that the soluble organic compounds that were formed furnished the substrate for bacterial metabolism, attempts were made to measure bacterial respiration. Streptomycin was added to plexiglass cylinders *in situ* that contained aquatic plants. The concentrations added resulted in a maximum of respiratory inhibition without decreasing photosynthesis. Differences in rates of respiration between treated and untreated cylinders were attributed to respiration of periphytic bacteria. These differences averaged to about 25 percent of the rate of community respiration. Assuming that all of the photosynthetically-fixed carbon is respired by the community, the 25 percent of community respiration that can be attributed to bacteria is very close to the 20 percent of photosynthetically-fixed carbon that escapes in the form of organic carbon.

MACROPHYTES OF THE FIREHOLE RIVER

Twenty-three species of aquatic plants were found in the Firehole River. Distribution and abundance of the species changed along the environmental gradient above, within, and below the geyser basins. Table III shows the occurrence of the species in various reaches of the river.

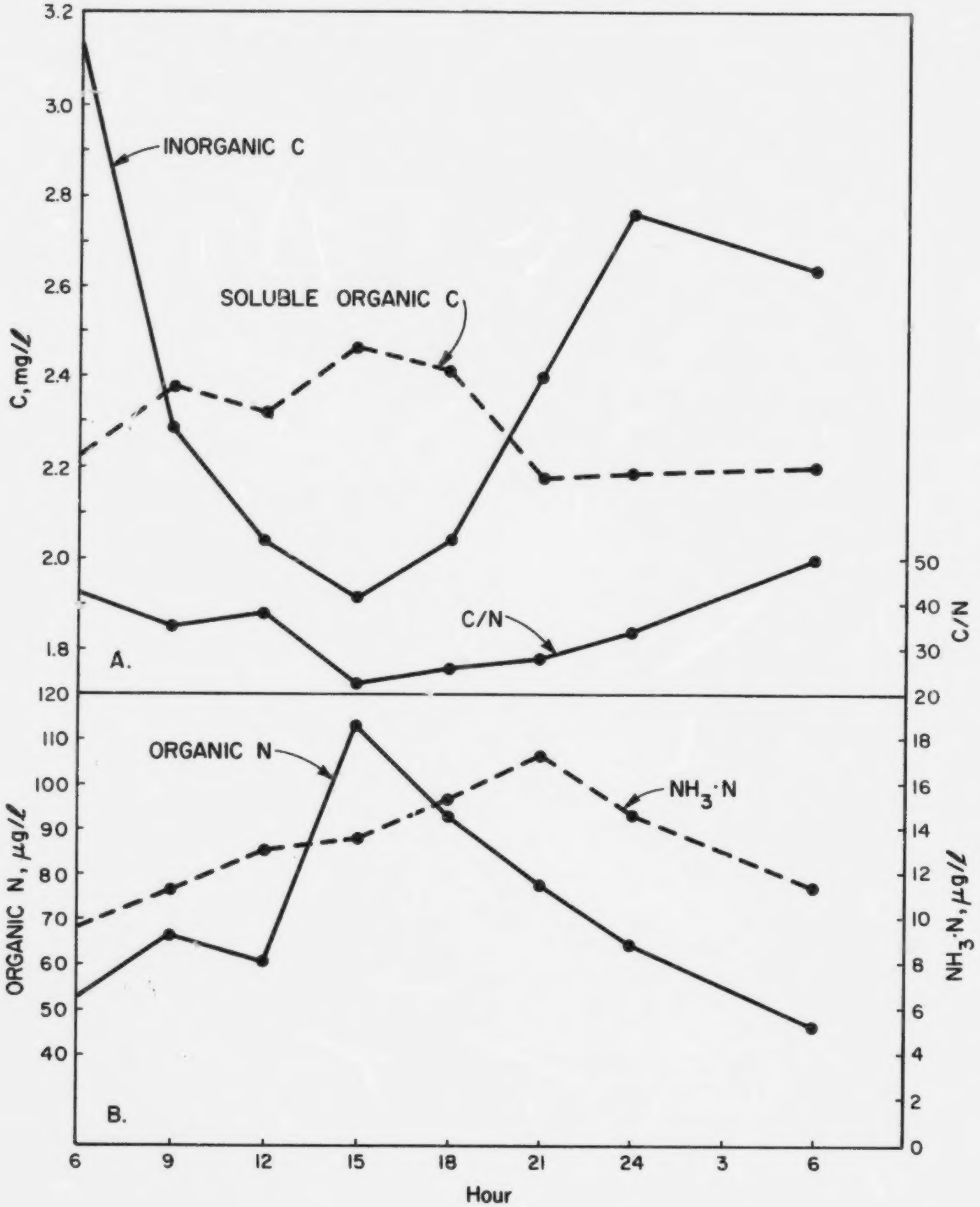

FIGURE 8. A. INORGANIC CARBON, SOLUBLE ORGANIC CARBON, AND THE CARBON/NITROGEN RATIO PRESENT AT VARIOUS HOURS IN THE MADISON RIVER ; B. AVERAGE ORGANIC AND AMMONIA NITROGEN

TABLE III

OCCURRENCE OF SPECIES OF AQUATIC PLANTS IN THE FIREHOLE RIVER

Plants	Location Relative to Geyser Basins				
	Above Basins	Upper Basin	Midway Basin	Lower Basin	Below Basins
Hippuris vulgaris	+				
Glyceria borealis	+				
Scapania undulata	+				
Ranuculus aquatilis	+	+			
Carex nebraskensis	+	+			
Fontinalis neo-mexicanus	+	+			
Berula erecta	+			+	
Myriophyllum exalbescens		+	+	+	+
Potamogeton strictifolius		+	+	+	+
Chara vulgaris		+	+	+	+
Potamogeton filiformis		+	+	+	+
Eleocharis acicularis		+	+	+	
Junus sp.		+		+	
Fontinalus antipyretica		+			
Rorippa nasturtium-aquaticum		+			
Hygrohypnum palustris		+			
Fissidens grandifrons			+		
Zannichella palustris			+	+	+
Nitella sp.			+	+	+
Ruppia maritima				+	+
Utricularia vulgaris				+	+
Potamogeton nodosus				+	+
Potamogeton pectinatus				+	+

Three species, (*Hippuris vulgaris*, *Glyceria borealis*, and *Scapania undulata*), were confined to the river reach above the Upper Basin.

Occurring only above or within the Upper Basin were *Ranunculus aquatilis*, *Carex nebraskensis*, and *Fontinalis neo-mexicana*.

Four species, that were also the dominant plants, were found in all of the basin reaches and also below (*Myriophyllum exalbescens*, *Potamogeton* *strictifolius*, *Chara vulgaris*, and *Potamogeton filiformis*).

Zannichellia palustris and an unidentified species of *Nitella* were found only in or below Midway Geyser Basin.

Four species (*Ruppia maritima*, *Utricularia vulgaris*, *Potamogeton vulgaris*, and *Potamogeton pectinatus*) occurred only in the Lower Basin reach and below.

Based on the dominant plants, two communities could be distinguished. A community characterized by *Glyceria borealis*, *Ranunculus aquatilis*, and *Fontinalis neo-mexicana* occupied upper river reaches where cooler temperatures and a lower dissolved salt concentration prevailed. The lower reaches with higher temperatures and higher dissolved solids were characterized by *Chara vulgaris*, *Potamogeton strictifolius*, *P. filiformis*, *Myrophyllum exalbescens*, and *Nitella* sp.

The percentage of river bed occupied by vegetation increased downstream. The results of a correlation analysis between plant cover, temperature, and total alkalinity are entered in Table IV.

TABLE IV

CORRELATIONS COMPARING PERCENTAGE COVER (1), TEMPERATURE (2), AND TOTAL ALKALINITY (3)

Simple Correlation Coefficients

$r_{12} = 0.550$

$r_{13} = 0.604^{a}$

$r_{23} = 0.896^{b}$

Partial Correlation Coefficients

$r_{12.3} = 0.025$

$r_{13.2} = 0.301$

Multiple Correlation Coefficient

$R_{1.23} = 0.607^{a}$

[a] Significant to 5% level.

[b] Significant to 1% level.

The simple correlation coefficient between plant cover and total alkalinity was significant to the 5% level. Partial correlation coefficients comparing cover to temperature and total alkalinity were not statistically significant. However, in view of the fact that the partial correlation coefficient between cover and temperature was virtually zero and since the multiple correlation coefficient was practically the same as the simple correlation coefficient between cover and total alkalinity, it would appear that total alkalinity was the more significant factor.

An index of diversity was calculated for each sampling station using a formula suggested by Margalef (1951).

$$d = \frac{(m-1)}{l_n N}$$

where m = number of species and N = number of plants.

When correlation coefficients were computed between diversity indices, temperature, and alkalinity (Table V), it was found that the simple and partial correlation coefficients between diversity and alkalinity were significant to the 1% level. Although the simple correlation coefficient between diversity and temperature was not significant, the partial correlation coefficient indicates a negative correlation between diversity and temperature significant to the 1% level.

TABLE V

CORRELATIONS BETWEEN DIVERSITY INDICES (1), TEMPERATURE (2), AND TOTAL ALKALINITY (3)

Simple Correlation Coefficients

$r_{12} = 0.473$

$r_{13} = 0.789^{a}$

$r_{23} = 0.896^{a}$

Partial Correlation Coefficients

$r_{12.3} = -0.860^{a}$

$r_{13.2} = 0.933^{a}$

Multiple Correlation Coefficient

$R_{1.23} = 0.946^{a}$

[a] Significant to 1% level.

PRELIMINARY STUDIES ON THE EFFECT OF CARBON SOURCES ON PHOTOSYNTHESIS BY VARIOUS SPECIES

Studies are now underway to determine the ability of the various plants to utilize bicarbonate ion in contrast to free CO_2. *Glyceria borealis* is found in the Upper Firehole above the basins and in the Gibbon River. These are habitats characterized by water of lower alkalinity and high free CO_2 content. *Myriophyllum exalbescens* is one of the dominant species of the Firehole River below the Upper Basin and also of the Madison River. It occurs in situations where the water has a higher alkalinity and lower free CO_2 content. Mean photosynthetic rates of these two plants are compared in Table VI.

TABLE VI

MEAN PHOTOSYNTHETIC RATES OF *Myriophyllum exalbescens* AND *Glyceria borealis* AT 13°C IN ALTERED OR UNALTERED RIVER WATER
(μℓ O_2/mg dry wt. /min)

Plant	River	Treatment	Relative CO_2	Relative HCO_3	Rate
M. exalbescens	Firehole	Unaltered	Low	High	0.0821
M. exalbescens	Gibbon	Unaltered	High	Low	0.0453
M. exalbescens	Firehole	CO_2 added	High	High	0.0865
M. exalbescens	Gibbon	CO_2 removed	Low	Low	0.0340
G. borealis	Firehole	Unaltered	Low	High	0.0072
G. borealis	Gibbon	Unaltered	High	Low	0.0271
G. borealis	Firehole	CO_2 added	High	High	0.0311
G. borealis	Gibbon	CO_2 removed	Low	Low	0.0053

Myriophyllum exalbescens apparently can utilize bicarbonate more efficiently than *Glyceria borealis* and seems to be less affected by the concentration of free CO_2. *Glyceria borealis*, on the other hand, seems to be dependent upon free CO_2 and possesses little ability to utilize bicarbonate. It is apparent that the physiology of these two species of plants is in accord with their ecological distribution.

DISCUSSION AND CONCLUSIONS

In spite of the fact that hard waters are more productive than soft waters, the limiting effect of CO_2 concentration on the rate of photosynthesis seems to be largely overlooked by limnologists. Birge and Juday (1911) long ago regarded the supply of carbon dioxide including the "half-bound" CO_2 in bicarbonate reserves as probably the chief controlling factor in productivity.

However, the present state of knowledge of carbon sources for various species of aquatic plants is highly unsatisfactory. Previous experimental results conflict, interpretations differ, and numerous perplexing questions remain unanswered (Sculthorpe 1967).

On the basis of laboratory experiments, Burr (1941) concluded that aquatic plants are adapted to low light intensities but not to low CO_2 tension. Steemann Nielsen (1944, 1946, 1947) studied the influence of the concentrations of carbon dioxide bicarbonate and carbonate ions on the photosynthesis of various aquatic angiosperms and the aquatic moss, *Fontinalis*. Both free carbon dioxide and bicarbonate ions were directly assimilated by *Myriophyllum spicatum* and other angiosperms whereas *Fontinalis* used only carbon dioxide. Carbonate ions were not available to either type. Photosynthesis of *Myriophyllum* was found to be independent of the hydrogen ion concentration in the range pH 4 to 10 and also independent of the ionic concentration when free carbon dioxide was the carbon source. However, with bicarbonate ion as the source, the photosynthetic intensity was very dependent on the absolute concentration of both cations and anions.

Results similar to those of Steemann Nielsen were obtained by Ruttner (1948) who found that whereas *Fontinalis antipyretica* used free carbon dioxide at a more or less constant rate but ceased to photosynthesize when the supply was exhausted, *Elodea canadensis* was able to continue, using carbonate ion as the carbon source. Examination of Ruttner's data shows that regardless of whether a plant can use bicarbonate, photosynthesis becomes rapidly rate-limited as free CO_2 tension decreases, which is in accord with the observations of Burr (1941).

Briggs (1959) suggested that in some systems where bicarbonate ions are absorbed, carbon dioxide is still the actual reactant and the effect of the bicarbonate ion is merely to increase the concentration of carbon dioxide at the sites of consumption, i.e., the chloroplasts.

On the basis of the field studies reviewed in this paper, it is concluded that maximal photosynthetic rates are attained only in flowing water of high total alkalinity (± 100 ppm $CaCu_3$). The percentage of stream bottom covered by vegetation and the diversity of the community also increases as the total alkalinity increases. However, regardless of whether free carbon dioxide or bicarbonate were used as a carbon source, the rate of photosynthesis decreased when initial pH values above 8.00 occurred. Presumably this is due to limitation of photosynthesis by decreasing concentrations of free carbon dioxide. Evidence is also presented suggesting that various species of aquatic macrophytes differ in their ability to utilize bicarbonate ion in contrast to free carbon dioxide. It has also been shown that, on the average, 20 percent of photosynthetically-fixed carbon appears in soluble organic form. The nitrogen cycle also seems to be involved, since the soluble organic substances become more enriched with nitrogen during photosynthesis and a rise in ammonia concentrations also occurs during the photosynthetic period. The suggestion was made that the soluble organic compound, released during photosynthesis, may be important as a food substrate for bacteria in the flowing-water ecosystems.

Odum (1956) has pointed out that high primary productivity is common in the early recovery zone of streams receiving effluents of high organic content because additional nutrients permit producers to make organic matter faster than it is utilized. Not only will such nutrients as phosphates and nitrates be in high concentrations in this zone but high CO_2 concentrations can be expected also. Enrichment by organic pollution of water with photosynthetically available CO_2 may be equally important to enrichment with other nutrients in causing eutrophication.

It also appears that the tendency of streams to become more productive downstream (Odum 1956) could not be sustained without continual accrual of available CO_2 by ground water, respiration, and decomposition of organic matter.

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TRACE ELEMENT MEASUREMENTS IN THE AQUATIC ENVIRONMENT

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INTRODUCTION

Findenegg (1966) aptly stated that the growth of plankton in lakes is controlled by the interaction of many factors which can be divided into three groups: 1) physical factors originating directly or indirectly from solar radiation, such as light conditions, temperature, mixing, and turbulence by the action of winds; 2) the content of nutrients in the euphotic zone of the lakes; and 3) the interaction of the organisms present in the plankton community which may promote or hamper the production of certain species.

The first and third groups by their nature lend themselves to fairly complete studies. A great deal is known about light, temperature, and turbulence in waters. Considerable information is also available regarding plankton cycles, numbers, and grazing factors. However, in the realm of nutrient study (aside from the major or macronutrients) a great deal of investigation is now desperately needed. Very little attention has been paid to micronutrients except in laboratory algal cultures. Notable exceptions are those investigations by Goldman (1960); Bachman and Goldman (1964); Goldman (1964); Schelske (1962); Goldman and Carter (1965); and Wetzel (1965). These studies were made in situ utilizing ^{14}C as a check on lake response to addition of a specific micronutrient.

One of the main reasons for lack of agreement among many other available papers may be due to contamination of cultural media, glassware, growth chambers, and collection equipment. In recent years, however, the quality of plastics, ion-exchange resins, field equipment, and labware have been improved to the point where simultaneous measurements of several micronutrients may now begin.

One of the most powerful tools available today for this type of study is that of neutron activation analysis. Earlier analytical methods were burdened by the tremendous task of chemical preseparation and concentration of elements in sub parts per billion (ppb) ranges. At these low levels, chance contamination by reagents, filters, glassware, techniques, and even laboratory airborne particles, if not nullifying the results, at least made them suspect. For example, materials leaching out of certain types of laboratory glassware may contain more of a particular trace element than a sample placed in the glassware for analysis. Hume (1967) points out that "pure" (distilled) water may contain Pb, Cd, and Cu in amounts of 10^{-8} to 10^{-9} M. He has suggested that it is not uncommon for laboratory workers to introduce additional impurities into a chemical reagent while attempting to further purify it.

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Robertson (1968) has discussed extensively the role of contamination in the ultra trace element analysis of sea water. He found relatively high quantities of trace elements in reagents, solvents, distilled water, polyethylene hoses, hydrowire, sampling devices, sample containers, membrane filters, and chelating agents. One of the more common contaminates in double distilled water was Ag, present in relatively high concentrations. Membrane filters surprisingly contained Cr and Zn along with lesser quantities of Sb, Co, Cs, and Fe. Borosilicate glass contained Fe, Hf, Sb, and significant amounts of Sc, Co, and Zn. Several chelating agents contained Fe, Zn, Cu, Ag, Sb, and Co.

Significantly high amounts of Zn and other trace elements are apparently present in Van Dorn type water samplers that utilize rubber end plugs.

Polyethylene bottles and vials, on the other hand, appeared to be relatively free from trace elements except Fe. Polyvinyl chloride (PVC) materials may contain Zn, Fe, and Cu, but Teflon seems to be relatively free from trace elements.

In view of the number of productivity studies, algae culturing experiments and bioassays now being undertaken, the researcher should be aware of a possible bias in his results that may be introduced by his equipment and reagents.

A tremendous advantage of activation analysis is that some 10 to 20 constituents present in varying quantities may be measured simultaneously in the same water sample. The analytical process is nondestructive and recounting can be done at anytime within the half life of the isotopes of the activated elements.

The great selectivity and sensitivity of neutron activation analysis is discussed further in the methods and instrumentation section of this paper. The important point to be made here is that with some care one can now measure almost the entire spectrum of micronutrients in a single 30-mℓ water sample taken directly from a field station. The high degree of accuracy exhibited by this method has been further enhanced this past year and even in the past few months by the replacement of sodium iodide (NaI) detection crystals with lithium drifted germanium detectors (Ge, Li). Figure 1 compares spectra of NaI and Ge(Li) detectors on the same water sample. The Ge(Li) crystals give much higher resolution than the previously utilized NaI system and at the same time eliminate the necessity for extensive pretreatment chemical separations.

This development precludes many contamination hazards in sample handling and also reduces the amount of time required for analysis.

Wide applicability of these detectors in the environmental field is now evident and will increase in the near future with the utilization of newer and larger crystal dectectors.

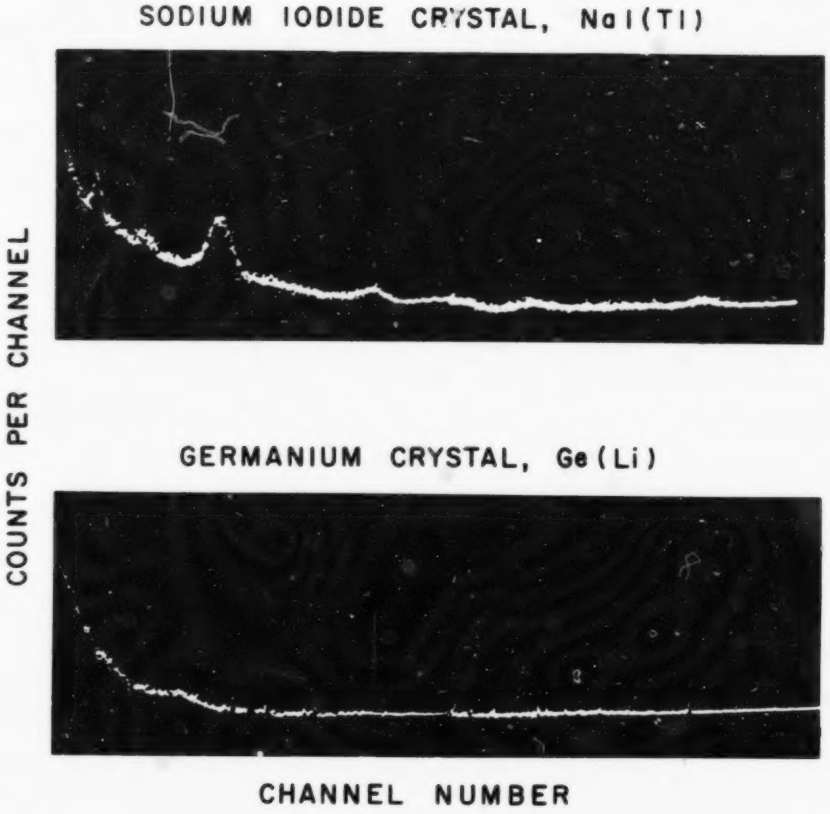

FIGURE I. COMPARISON OF RESOLUTION OF NaI AND Ge(Li) DETECTION SYSTEMS (GAMMA RAY SPECTRA OF WILLIAMS LAKE WATER)

The initial objective of this study was to investigate methods of direct measurement of trace elements in the aquatic environment of lakes and streams with a minimum of chemical preseparation. A second consideration was to measure, if possible, trace element concentration by algae.

In attempting to achieve these objectives we selected two lakes (Rock and Williams) located in the basaltic channeled scablands of the Columbia plateau (Bretz 1959). Both lakes are narrow, long, and subject to considerable wind action. Depths vary from 1 to 33 meters on the average and over 100 meters in some portions of Rock Lake. Williams Lake has been subjected to some added nutrients from recreational uses while Rock Lake receives its additions from farm surface runoff. However, until now these eutrophic pressures have been light to moderate. Limnological measurements, including macronutrients, have been regularly made for three years. These data indicate that Rock Lake has sufficient macronutrients available for algal blooms but so far none have occurred.

Williams Lake, on the other hand, has shown increased blue-green algal growth each year during the late summer. In 1968 a short lived Aphanizomenon flos-aquae bloom occurred during September.

METHODS AND INSTRUMENTATION

Determination of Cr, Fe, Co, Sc, Zn, Sb, Cs, Rb, Au, Th, Na, Hg, and U

Samples of water or algae were collected in 30-mℓ Nalgene polyethylene bottles which had previously been leached with double distilled HNO_3 followed by double distilled water rinses. After collection the bottles were sealed, placed in dust free polyethylene bags, and transported to the reactor laboratories. These samples and appropriate standard solutions (also in bottles) were then irradiated in the Washington State University – TRIGA – III – Type research reactor for 8 hours at a neutron flux of 5×10^{12} $n/cm^2/sec$. The samples and standards were rotated about a vertical axis during irradiation to insure neutron flux homogeneity.

After irradiation the bottles were removed from the reactor core and placed in HNO_3 for 6 hours to remove surface impurities which may have been adsorbed during irradiation. Two days after irradiation the samples were counted to determine the ^{24}Na activity. Standards made up for each element had exactly the same geometry as the samples. After ^{24}Na had decayed to negligible levels (10-14 days) the samples were again counted for 20,000 to 40,000 seconds in the case of the water and 2,000 to 10,000 seconds in the case of the algae samples. A blank polyethylene bottle was irradiated and counted to determine the contribution of the bottles to the activities of the elements determined.

Peak areas for each radioisotope to be determined were measured using standard Ge(Li) spectroscopy techniques. Decay corrections were applied so that the activities of the samples and standards were calculated for the same decay time after irradiation. Nuclear characteristics of the radioisotopes of the elements measured are shown in Table I.

Determination of K, Sr, Ba, Br, Mn, and Cu

Samples and standards were irradiated for 3 hours and allowed to "cool" for 30 minutes. To each sample an equal volume of HNO_3 was added. One gram of Sb_2O_5 (RAl) ERBA was added to the solution and agitated for 20 minutes. An additional 1 g Sb_2O_5 was added and agitation continued for another 20 minutes. The mixture was then centrifuged and the supernatant transferred to a 60-mℓ bottle. The residue was washed with 8M HNO_3 and the washings added to the original solution. This procedure effectively removes the ^{24}Na allowing ^{42}K, ^{64}Cu, ^{56}Mn, ^{87m}Sr, ^{139}Ba, and ^{82}Br to be determined. Figures 2 and 3 show gamma ray spectra before and after removal of ^{24}Na.

TABLE I

NUCLEAR REACTIONS AND GAMMA-RAY ENERGIES

Element	(n, y) Product	Half Life	Gamma Ray Used (MeV)
Sodium	^{24}Na	15.0 h	1.37
Manganese	^{56}Mn	2.58 h	0.845
Copper	^{64}Cu	12.8 h	0.511
Gallium	^{72}Ga	14.1 h	0.835
Lanthanum	^{140}La	40.2 h	1.60
Chromium	^{51}Cr	27.8 d	0.320
Scandium	^{46}Sc	83.9 d	0.889
Cobalt	^{60}Co	5.26 y	1.173
Barium	^{131}Ba	12.0 d	0.374
Antimony	^{124}Sb	60 d	1.70
Gold	^{198}Au	64 h	0.411
Iron	^{59}Fe	45 d	1.098
Thorium	^{233}Pa	27.4 d	0.316
Zinc	^{65}Zn	245 d	1.112
Cesium	^{134}Cs	2.1 y	0.603
Rubidium	^{86}Rb	18.7 d	1.078
Potassium	^{42}K	12.4 h	1.525
Bromine	^{82}Br	36 h	0.554
Mercury	^{203}Hg	46.6 d	0.279
	(n, f) Product		
Uranium	^{141}Ce	32.5 d	146 KeV

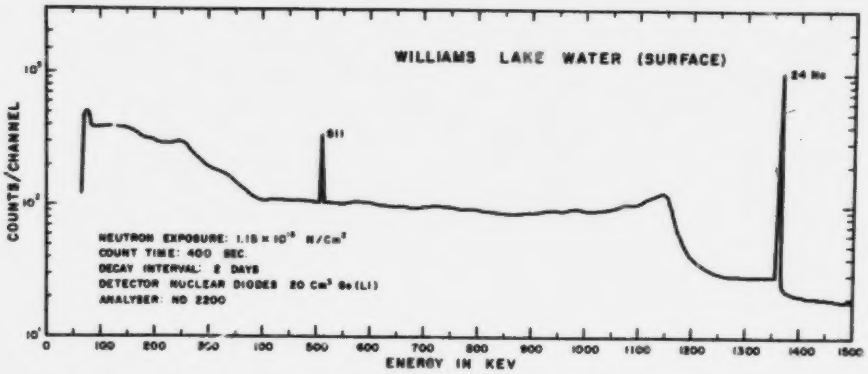

FIGURE 2. WILLIAMS LAKE WATER SHOWING ^{24}Na PRIOR TO ITS REMOVAL BY ADSORPTION BY ERBA RA-1

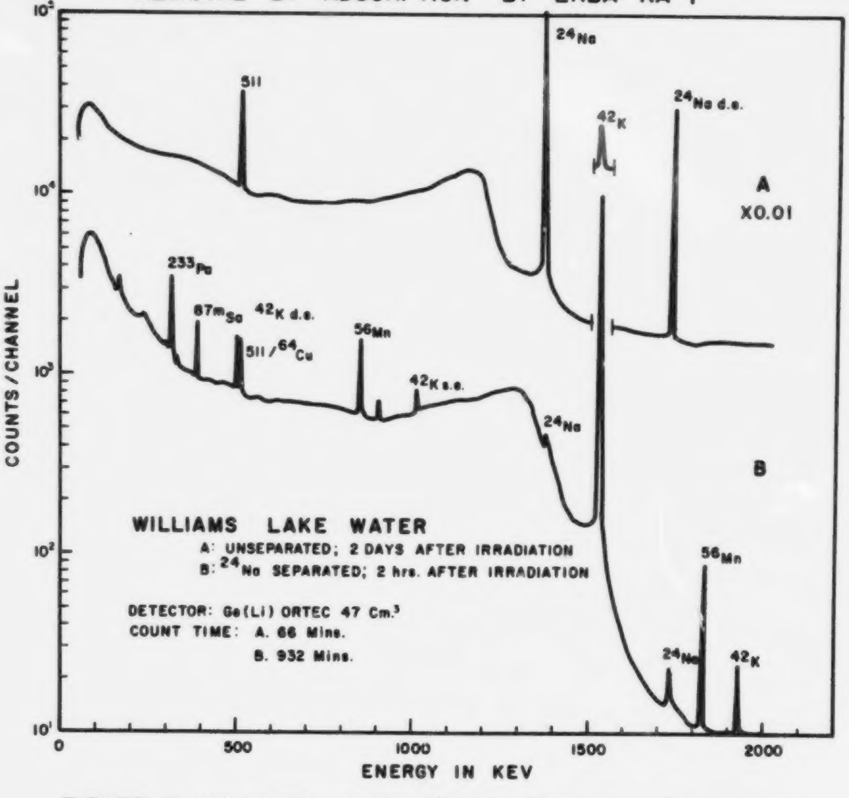

FIGURE 3. WILLIAMS LAKE WATER BEFORE AND AFTER REMOVAL OF ^{24}Na INTERFERENCE

Calculations

The following formula was used to determine the weight (in μg) of each element in the sample.

$$\text{Weight in micrograms} = \frac{\text{Weight of element in standard used } (\mu g)}{\text{count rate of standard}} \times \text{count rate of sample} .$$

Instrumentation

A large (47 cc) Ortec Coaxial Ge(Li) detector was utilized in conjunction with an Ortec 118 preamplifier and 435 amplifier. A nuclear data ND2200 4096 gamma channel analyzer with a digital printout provided the data collection system.

For 1.33 MeV gamma, the photo peak area obtained by the 47-cc Ge(Li) detector was 9 percent of that obtained by a 3 x 3 in. (7.6 x 7.6 cm) NaI system. This figure indicates the high resolution of the Ge(Li) system. The ratio of peak height to Compton background was 20:1. Resolution was 2.9 KeV at 1.33 MeV.

RESULTS AND DISCUSSION

We have been able to demonstrate that trace element samples obtained in the field can be brought directly to the nuclear reactor, activated, and counted with minor, and in some cases no, preseparation. This is somewhat in contradiction with Hume's (1967) statement that it would be some time before this method (activation analysis) could be applied to field conditions. It should be mentioned in all fairness that research reactors of this type do not exist in all areas. The point to be made, however, is that by utilizing the same bottle that the sample is collected in, transporting it in a dust free polyethylene bag to the reactor, and then activating it certainly offers excellent protection from contamination.

An inventory of contaminates in the polyethylene bottle, double distilled nitric acid and water can easily be made by activating one or several of the bottles treated in the same manner as the sample. The trace elements in the blanks may then be subtracted from the gross amounts measured in the samples.

It was shown during our course of study that several elements adsorb readily to the walls of polyethylene containers. This is especially true of Zn, Au, and Sb. After activation and counting one can empty the water, return the bottle to the counter, and make a second "hot" count. No doubt this helps to explain the disconcerting lower values found when an investigator retests a water sample for the same element after it has been stored for a period of time. Heron (1962) for example, has written on the loss of phosphorus in polyethylene containers after storage.

We first discovered a serious loss of metallic ions when we were originally transferring our field samples from collecting bottles to other bottles for activation. In later attempts we acidified the collecting bottles to counteract this "sorption." Other workers have predicted or experienced similar problems.

Robertson (1968) has described a situation where the opposite reaction has occurred. In this instance a Van Dorn type sampler was allowed to hold water for an hour after which a subsample was taken and activated. An increase of 10 µg/ℓ Zn was measured over the amount determined in the original water sample.

Preseparation techniques, as modified by Filby et al. (1969a), seem to have overcome the problem of ^{24}Na interference when testing for K, Cu, Mn, Sr, Ba, and Br. This method appears to be precise and sensitive with negligible contamination by either reagents and/or manipulation.

Lake Water Analysis

The data from activation analysis of lake waters are presented in Table II. An attempt was made to sample widely separated areas as well as depths in the two lakes. Samples for depth analysis were taken in the same area in Williams Lake for each sampling period as shown by Table II. The first period was July 19, 1968 just after the cessation of the last diatom pulse of the summer. The waters were fairly free of plankton. Total counts of *Fragilaria crotonensis*, *Stephanodiscus niagarae*, and *Ankistrodesmus falcatus* were less than 20 cells/mℓ. The measurements in Table II indicate relatively uniform trace element distribution throughout the lakes with some increase in trace elements in the hypolimnion. In Rock Lake this condition was somewhat reversed in the case of Fe. Both lakes were well stratified during this period but were not devoid of dissolved oxygen. In fact, the lowest measurements recorded (2.1 to 3.0 mg/ℓ) were in the hypolimnion of Williams Lake in late August 1968.

Samples taken on March 11, 1969 revealed a higher trace element content. These samples were taken through holes cut in the ice cover. Plankton numbers were only 1 to 2 cells/mℓ in all counts made. Total suspended solids ranged between 3 and 5 mg/ℓ and volatile solids were less than 1 mg/ℓ. Dissolved oxygen ranged between 4 to 6 mg/ℓ.

Rock Lake was not covered by ice at this time, but plankton counts were no higher than Williams Lake. Trace elements in Rock Lake in most cases were several magnitudes higher but total suspended solids were also higher (25.4 mg/ℓ) with volatile suspended matter ranging between 0.7 to 0.9 mg/ℓ. Dissolved oxygen occurred in amounts of 8.0 to 10.0 mg/ℓ.

TABLE II

LAKE WATER ANALYSIS

(Elements are listed as μg/ℓ unless otherwise noted)

Station	K mg/ℓ	Fe	Mn	Cu	Cr	Co	Sc	Zn	Sr	Br	Sb	U	Au	Ba	Hg	Cs	Th	Rb
Williams Lake																		
Area A	7.10	187.0	23.7	1.78	2.37	0.20	0.03	11.9	7.53	21.8	0.13	2.83	0.07	43.9	5.35			
Area B	5.70	94.3			2.43	0.18	0.04	13.3			0.18	2.81	0.09	16.5	5.13			
1.0 Meter					2.25		0.02	38.2			0.15	1.62	0.08	18.9	3.43			
Hypolimnion		1970.0			5.46	1.04	0.37	124.6			0.37	23.70	0.15	143.9	3.70			
Rock Lake																		
Epilimnion	4.57	1078.0	1 7	1.92	3.94	0.46	0.03	31.1	120.80	30.4	0.37	7.31	0.10	104.1	4.48			
Themocline		587.0				0.47	0.04	50.4			0.93	7.30	0.10		1.07			
Hypolimnion		301.0				0.40	0.00	115.1			1.23	18.00	0.15	297.0	1.15			
Williams Lake (under ice)																		
Area A		1.3			07.15	0.41	0.38	340.8			07.78		0.18			0.5	0.1	9.78
Inlet		27.9			09.48	0.98	0.68	158.4			01.59		0.01			0.1	3.5	
Bottom		36.3			12.41	0.41	0.83	190.5			01.38		0.06			0.2	610.4	18.49
Rock Lake					08.83	34.68	0.63	241.7			08.33					0.1		45.07

Algae Trace Element Analysis

Direct activation analysis had been made up to this point without attempting to remove particulate matter, including algae. The next step was to attempt to separate algae from the water in which it was growing and then to activate and count both samples. Water from Williams' Lake (March 19) was centrifuged in a polyethylene tube decanted into a bottle and then activated. The resulting spectra shown in Figure 4 were almost identical to uncentrifuged samples of the same station due to very low suspended matter.

Filamentous algae (Spirogyrea sp.) were recovered from the shoreline of Rock Lake and centrifuged in the same manner. The liquid portion was decanted into another bottle and both bottles were then activated and counted. The results (shown in Table III) were somewhat surprising in the relatively high concentration of many of the trace elements. According to Weissner (1962) two workers, Knauss and Porter, have found that certain trace elements were concentrated by algae in direct proportion to their abundance in water. He also suggests that some algae may bind cations by acidic polysaccharides in their cell walls. Our first thoughts were that the algae might be acting as filters or catchers for microscopic dirt or rock particles. Subsequent activation analysis of the rock substrata indicate that this may be the case. The algae and water spectra are shown in Figure 5.

After initial separation we centrifuged the algae subsamples several times and resuspended them in double distilled water. This procedure probably gets rid of much of the soluble material associated with the algae cells without a great deal of contamination. Particulate matter adhering to the algal cells and filaments may well be a different matter. Differential centrifugation has been offered as a suggestion and no doubt it will be tried in the near future. It might also be helpful to activate the total sample first and then follow with algae separation. This would eliminate corrections for distilled water and at least one sample bottle.

Similar problems were encountered in sampling the Palouse River which receives heavy flows of sewage from two cities (Figure 6). Both Cladophora sp. from the Palouse River and Scenedesmus quadricauda from a laboratory culture show high "sorption" of Fe, Zn, and Cr. It was also interesting to note the great variety of trace elements in the laboratory media (Allen 1952) which had been prepared from reagent grade chemicals, a hard glass still, and a hard glass growth chamber. No doubt all of these items made their own contribution.

In polluted streams it appears that algae have a tendency to concentrate or "adsorb" very high quantities of trace metals. A great deal has been written about the possibility of organic acids, algae, and microorganisms complexing Fe, Mn, and other metals. Shapiro (1964), Hutchinson (1957), Wiessner (1962), Fogg (1953), and Perfil've (1964) have all written on this subject. In addition, Ives (1959) has shown that algae exhibit a negative charge. It could well be that algae exert a far greater influence on the nutrition of a lake or a stream than has been realized. It may be possible that they act as flocculating agents in settling nutrients out of the water. They may alternately take up and release metals to the water, or possibly act as chelating agents complexing many of the metals and keeping them in solution.

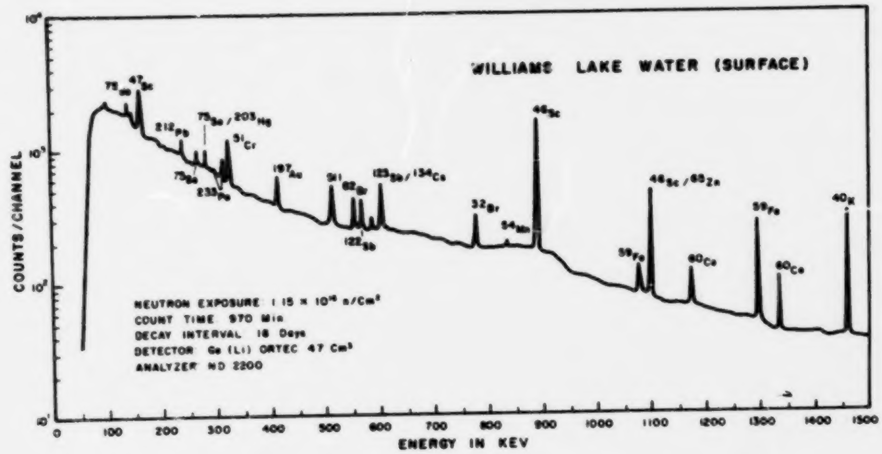

FIGURE 4. WILLIAMS LAKE WATER AFTER REMOVAL OF SUSPENDED MATTER BY CENTRIFUGATION

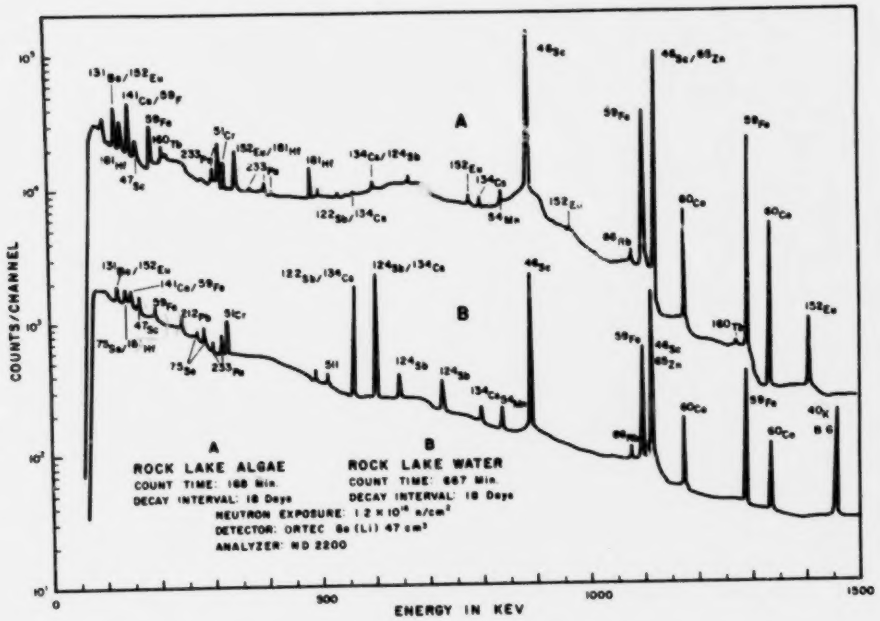

FIGURE 5. COMPARISON OF CONCENTRATIONS OF TRACE ELEMENTS IN WATER AND ALGAE FROM THE SAME SAMPLE

TABLE III

TRACE ANALYSES OF ALGAE AND THE WATER FROM WHICH THEY WERE TAKEN

(Elements are listed as μg/g)

Identification	Fe	Mn	Cu	Cr	Co	Sc	Zn	Sr	Br	Sb	U	Au	Ba	Hg	Cs	Th	Rb
(Rock Lake)																	
Spirogyrea sp.				29.83	8.04	8.51	204.96			159.68		9.68			1.51	02.6	15.40
Spirogyrea media	1.50			7.28	01.10	0.38	187.30			0.64		0.00			0.10	0.1	4.15
(Palouse River)																	
Cladophora sp.				175.12	60.67	224.74	975.32			150.90		70.33			2.71	01.8	45.86
Cladophora media	4.67			22.90	01.83	3.00	415.38			0.68		0.65					55.00
Scenedesmus quadricauda	270.50			29.34	034.60	15.53	210.06			119.58		0.40			4.80	5.6	35.00
Scenedesmus media	1.27			01.70	08.80	3.07	434.83			03.63		0.10			1.30	0.2	06.26

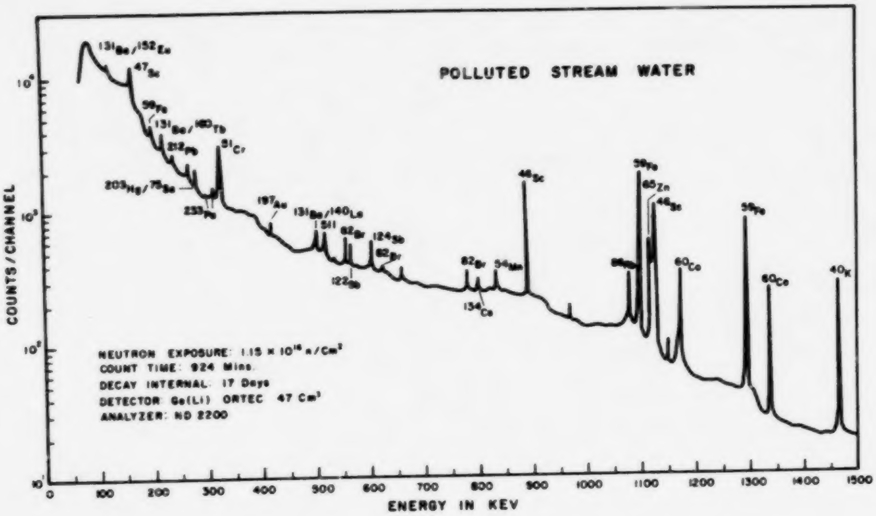

FIGURE 6. TRACE ELEMENTS IN WATER FROM PALOUSE RIVER
(A SEWAGE POLLUTED STREAM)

In summary it may be said that activation analysis appears to be suitable for direct study of the aquatic environment. The procedures developed here could be initially utilized to survey lakes and streams to delimit the micronutrients available. It would then be a much easier task to test for algal responses to the addition of specific micronutrients (since it would be known which were abundant and which were lacking). The responses could then be measured by the ^{14}C or other suitable methods.

At this·time one cannot speculate on the nutritional requirements or the trace element composition of the algae tested in this study because of the limited number of analyses made and the difficulty in eliminating the particulate matter adhering to the algal cells.

When enough data are gathered and procedures become standardized, it might then be possible to determine patterns of algal growth that are directly related to either over abundance or lack of certain trace elements.

ACKNOWLEDGMENTS

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EUTROPHICATION CONTROL AND APPLICATIONS SESSION

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BIOSTIMULATION AND TOXICITY CRITERIA AND APPLICATIONS IN DESIGN – PRESENT PRACTICE AND FUTURE POSSIBILITIES

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INTRODUCTION

In general terms the magnitude of a water quality problem can be defined as the difference between desired water quality and the perceived water quality. Although this is a seemingly simple definition, the basic problem of controlling water quality is exceedingly complex. Part of the complexity is due to the fact that both desired and perceived water quality are not fixed, but are variables changing with time, location, and circumstance. In quantitative terms, water quality conditions, desired or perceived, can be expressed as multidimensional vectors where each characteristic or water quality parameter constitutes a dimension and the quantitative measure of that parameter is one component of the vector. The goal of any water quality control program is to reduce the difference between the desired water quality vector and the perceived water quality vector to some "acceptable" level.

The parameters defining the dimensions of desired water quality are constantly changing; the trend is toward an increasing number of parameters to be considered. This has come about in part through increasing awareness of the factors affecting water quality, greater use of our water resources, and an increasing standard of living which has made more resources available for what used to be considered nonessential expenditures. All of these factors have added new dimensions to our definition of desired water quality. Increasing technical knowledge has permitted quantitative expression of many parameters that formerly could be expressed only in general, qualitative terms.

Increasing analytical ability and availability of resources for water quality studies have resulted in drastic changes in our perception of water quality. This trend, also, has been in the direction of greater complexity.

These points are clearly demonstrated by the current attention and research in the areas of biostimulation and toxicity. In the recent past the principal water quality problems were considered to be those resulting from discharges of oxygen-demanding substances and settleable solids. Great effort was expended to understand dissolved oxygen relationships in receiving waters and to improve the design of waste treatment plants for the removal of solids and biochemical oxygen demand. To a large extent these technical problems are fairly well solved. Now we have moved on to concern for the aesthetic characteristics of receiving waters and for protection of aquatic biota.

Current efforts with respect to both biostimulation and toxicity are directed in two areas; first, research to develop an understanding of the mechanisms involved, and second, development and improvement of methods to control the adverse effects of these two added dimensions of the water quality vector.

Unfortunately, as is usually the case, the impetus to study these questions is the increasing awareness of serious current water quality problems. Unexplained fish kills, diminishing shellfisheries, and advanced eutrophication of surface waters in many areas point to the need for immediate solutions if irremediable damage to our resources is to be minimized. The purpose of this brief paper is to suggest a rational approach to setting criteria for biostimulants and toxicity that can be implemented rapidly in spite of limited understanding of the fundamental biological processes involved.

Toxicity and biostimulation are terms used to describe two aspects of biological growth response. Toxicity results in inhibition of growth — usually the term is used in the context of inhibition of the growth of desirable organisms. Biostimulation results in enhancement of growth — and the term is usually applied to the excessive growth of undesirable organisms. Since both toxicity and biostimulation are concerned with organism response, it is possible that similarities may exist in methods of determining and expressing criteria for their control.

TOXICITY

At the present time standards and criteria intended to control toxicity in waste discharges usually are expressed either as blanket prohibitions against the discharge of toxic materials, i. e., substances harmful to the biota, or as maximum receiving water concentrations of specific toxicants. In a very few instances toxicity criteria have been stated as some fraction of a median lethal tolerance limit (TL_m).

Current investigations to develop toxicity criteria are most often aimed at extending the list of specific toxicants and estimating the allowable or "safe" concentrations of specific toxicants in receiving waters. These investigations are needed to improve our understanding of the toxication processes, but they are not sufficient to protect the receiving waters for several reasons. The behavior of toxicants varies greatly with the physical and chemical characteristics of the receiving water and from organism to organism. Thus, even for presently known toxicants the number of combinations of organisms, toxicants, and environmental conditions is staggering. To further complicate the problem, new toxicants are being constantly created at an alarming rate. As a result, control of toxicity by specification of individual toxicants is an unwieldy and unsatisfactory tool for either design or enforcement.

Much thought and writing has gone into the subject of determining application or "safety" factors to specify toxicity criteria. This method traditionally has been to measure toxicity in terms of the batch type, acute or lethal bioassay to determine a TL_m and then to specify the allowable concentration in the receiving water by reducing the TL_m by some arbitrary fraction or application factor. Typical application factors that have been used range from 0. 1 to 0. 01. This procedure has the advantage of being easy to apply, but it is not rational in that the application factors are seldom related to what we are trying to control, environmental response. The primary, measured response is the rapid death of the test organism, and arbitrarily reducing the concentration that causes this response is no guarantee of the continued health or survival of the organisms.

A number of alternative procedures have been proposed and used to overcome this well recognized problem. One approach has been to develop sublethal bioassay procedures where the primary response measured is some organism response short of mortality. Response variables that have been used include respiration rate, gill movement rate, flagella movement rate, heartbeat rate, acetylcholine levels, egg viability, and learning rate. These types of bioassays have the attraction of measuring a parameter perhaps more nearly related to long-term health of the organism, but even here the response selected may not be relevant to the problem of the health of the total biological community.

Another approach has been to extend the bioassay period well beyond the normal one to a four-day test period. Work currently underway at the Federal Water Pollution Control Administration's National Water Quality Laboratory in Duluth, Minn., is concerned with measuring organism response over its entire reproductive cycle, from egg to egg, so to speak. This procedure, for the particular organism being studied, is closer to measuring what we are trying to protect than the standard, acute bioassay. The disadvantage of this method is that it is lengthy, expensive, and measures the effects on one test species under one set of environmental conditions. It is probably impractical as a design or enforcement tool. Its greatest value lies in extending fundamental knowledge and in providing a better base for determining application factors to be applied to the results of more rapid, simpler tests.

The need for immediate control of toxicity in wastewaters is becoming increasingly apparent. Clearly there is a need for practicable procedures to establish toxicity criteria related as directly as possible to maintenance of a healthy aquatic biota.

Studies conducted over the last ten years at the University of California at Berkeley and more recently in the San Francisco Bay—Delta Program have resulted in an approach to establishing toxicity criteria based on measures of biological community structure. In brief, the method is based on relating the quantities of toxic materials discharged in an area to presumed alterations of the community structures.

The University of California studies developed a large mass of data on the toxicity of municipal and industrial wastewater discharges to San Francisco Bay. These data were derived from the results of acute, 96-hr bioassays using a fish indigenous to the Bay. To quantitate the amounts of toxicity entering the bay from waste discharges, a parameter called Relative Toxicity (RT) was defined for a particular waste discharger. Relative Toxicity is defined as the volumetric flow rate of the discharge divided by the 48-hr TL_m as determined in a series of bioassays. Thus, the TL_m, or rather its reciprocal, is used as a weighting factor to be applied to the discharge flow rate. In this fashion volumetric emission rates of toxicity are determined. The next step is to apply suitable calculation or modeling techniques to estimate the concentrations of RT at each point in the receiving water.

The University of California studies had also collected a vast amount of data on the types of phytoplankton, zooplankton, and benthic fauna in

San Francisco Bay. Because of their importance in the food chain and relative community stability, the benthic communities were analyzed exhaustively. A quantitative measure of species diversity (species diversity index) first proposed by Margalef was used as one measure of community structure.

The remainder of the analysis was based on the following two assumptions:

1. For a given location and normal (i. e., unpolluted) environmental conditions there is a "normal" or natural community structure with an associated "natural" species diversity.
2. Environmental stress (i. e., the presence of toxicants) will affect the community structure by eliminating more sensitive species and thereby reducing the species diversity.

In the San Francisco Bay it was found possible to predict quantitatively the natural or expected species diversity indices. Predicted indices were compared with indices calculated from samples collected in the Bay. In most cases the observed diversity indices were lower than the predicted indices and the magnitude of the difference in the two was closely related to calculated concentrations of Relative Toxicity at the sampling locations. The results of this analysis showed that increasing concentrations of Relative Toxicity were associated with depression of the benthic animal diversity indices below predicted values. The concentration of Relative Toxicity corresponding to a statistically significant change in the species diversity was 40 ml/ℓ for the southern reach (south of the Golden Gate) of the Bay.

Thus a quantitative toxicity criterion was established directly related to an observed ecological effect. The technique does not, of course, depend on using acute bioassays to measure toxicity; sublethal or extended bioassays could also be used.

BIOSTIMULATION

The similarity between approaches to, and problems of, setting toxicity criteria and those of establishing biostimulation criteria is striking. Current research efforts are oriented primarily toward the identification of specific growth-stimulating substances and the determination of allowable or safe concentrations in receiving waters. In this area the problems are similar to those outlined above for toxicity. There is a vast array of present and potential biostimulants, their effects are strongly dependent on environmental conditions and their effects vary greatly depending on the particular organisms of concern. Several recent studies have shown that in particular situations the addition of wastewaters to receiving waters resulted in growth rates that could not be accounted for in terms of the commonly considered nutrient constituents.

Clearly the present state of knowledge does not provide assurance that the effects of biostimulation can be controlled by setting maximum allowable concentrations for specific biostimulants. However, accelerating eutrophication of surface waters in many areas requires that we find a practicable means of setting effective criteria for design and enforcement purposes.

The following proposed procedure is analogous to the one described above for toxicity criteria. In brief, it consists of measuring the gross biostimulatory characteristics of a wastewater stream, converting this information to calculated concentrations in the receiving water, and relating these concentrations to observed environmental responses.

The work required would consist of the following elements:

1. For a given receiving water, identify potential biostimulant sources such as municipal and industrial wastewaters, agricultural drainage, runoff, etc.

2. Conduct bioassays using indigenous algal species and various wastewater—receiving water mixtures under conditions of light and temperature similar to those found in the receiving water. The measured bioassay response parameter would be some specified increase (say 50 percent) in the observed growth rate of the test organisms. The reported bioassay result would be the percent or concentration of wastewater in the culture corresponding to the specified response.

3. A new parameter, Relative Biostimulant Content (RBC), would be defined as the waste discharge flow rate divided by the wastewater concentration determined from the bioassay procedure. This would provide a measure proportional to the total biostimulants contained in the wastewater. As such it is directly analogous to the concept of Relative Toxicity.

4. In the receiving water itself, determine in situ growth rates and standing crop and characteristics of algal populations throughout the year.

5. Calculate concentrations of RBC at the various sampling stations throughout the receiving waters.

6. Determine the relationship between RBC concentrations and algal population changes using regression techniques.

A defined, quantitative relationship between calculated RBC concentrations and algal response in the receiving water would provide the information necessary to set a numerical total biostimulant criteria corresponding to selected algal population changes. Such a tool would also permit evaluation of the efficiency of wastewater treatment processes in removing biostimulants, not in terms of specific constituents such as nitrogen and phosphorus, but in terms of environmental response.

It is not suggested that a criterion such as proposed here would negate the need for research in the effects of specific biostimulatory constituents. It would supplement these investigations. Indeed, it would be extremely useful as a survey and screening tool to identify specific problem sources for more intensive investigation.

THE USE OF REMOTE SENSING IN WATER RESOURCE MANAGEMENT

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INTRODUCTION

Today the general public is becoming aware of the widespread economic significance of water resources and of the necessity to use these resources wisely. Water pollution is recognized as one of the most important problems facing our nation. Legislation has been initiated to construct vast networks for water transportation and storage, and to protect the freshwater resources demanded for agriculture, industry, recreation, and municipal uses. The necessity of feeding the hungry peoples of the world has led to experiments in harvesting the resources of the sea.

Seven-tenths of the earth's surface is covered by water, and all of man's cities and industries are located with respect to adequate water supplies. Ninety percent of the population of California lives within the coastal zone, which constitutes eight percent of the land area (Bendix 1968). Thus, it is obvious that to manage properly the water resources of the world and ensure mankind of a continued supply of freshwater, accurate and timely information must be available on 1) location of the water resources, 2) volume of the water supply, 3) condition of the water, and aquatic plants and animals, 4) condition of the channels, shorelines, and beaches, 5) condition of the watershed soils and vegetation, 6) potential flood problems and damage to channels, land, and property by floods, and 7) location and condition of harvestable plants and animals in the streams, lakes, and oceans.

Application of Remote Sensing to Water Resource Management

Existing information on our ocean and freshwater resources is grossly inadequate. This information could be increased by means of improved inventories and resource analyses in all of the major water resource areas of the world. To achieve this, data must be acquired from many sources including that of the synoptic aerial view provided by remote sensing as well as the microscopic view provided by comprehensive laboratory analysis.

Remote sensing is defined as the acquisition of information about an object that is not in intimate contact with the information-gathering device. Remote sensing of an area provides an overall view not obtainable by any other means. The energy recorded by remote sensing can be selected as to spatial, spectral, and temporal characteristics, and change in conditions can be evaluated by comparing several records of the same scene. The results of an activity or engineering project can be observed by periodically recording the scenes involved. Remote sensing has been found to be very useful in natural resource exploration.

Marine Resources

Much more information is available on our freshwater resources and inhabitants than on the marine environment. In his book, Marine Botany, E. Yale Dawson sets the stage for remote sensing investigations of the sea (Dawson 1966).

> "The sea is everywhere around us. Literally, one can now cross it between meals, and the three oceanic shores of the United States can all be visited in a day's time. The astronauts see the world in a new perspective, as a watery planet with but minor patches of land. Indeed, the oceans are really all one, divided more or less into five regions by the great continental islands. We call these oceans by separate names--the Pacific, and Atlantic, and Indian, the Arctic, and the Antarctic. Yet they are a continuous 71 percent of the surface of the earth, and there is more to them than meets the eye, for they are mostly deep and dark.
>
> "The oceans have an incredible average depth of more than three miles. We know from wide exploration and sampling by oceanographic expeditions that living things are present throughout all of them, to the greatest depths of 10,000 meters; but we also know that, except for a minute surface fraction, the whole of the enormous marine environment is utterly dark and cold. The creatures that make it their home never know sunlight nor temperatures much above freezing. This preponderant darkness has a profound effect on the life of the ocean, for in the absence of light, plants, except for obscure fungi and bacteria, are essentially lacking, and the vast, dark spaces of the sea are almost the exclusive realm of animals.
>
> "Sunlight falling upon the sea surface penetrates the water to varying depths, depending upon the intensity and angle of radiation, the amount of surface reflection, and the transparency of the water. The sea has exceptional light-absorbing capacity, so that even in quite clear water only some 35 to 38 percent of the sunlight that enters the water reaches a depth of one meter. With increasing depth the light quickly fades, and only a fraction of one percent remains below 50 meters. In coastal waters, in which sediment or plankton produce a turbid condition of low transparency, 99.5 percent of the light may be absorbed in the first 10 meters. Light is not only rapidly but selectively absorbed, so that the longer red, violet, orange, and yellow wavelengths are removed first, leaving only the shorter blue and green for deeper penetration. Thus, color below the surface is not in our familiar pattern, and the underwater observer must learn a new set of values in order effectively to interpret what he sees."

This last statement applies as well to the aerial observer as he looks into the near surface areas of the sea.

Freshwater Resources

Much has been written about the problems related to acquiring freshwater supplies for cities, industries, and recreation, and to enlighten the public about water pollution problems. Therefore, a comprehensive discussion is not presented here.

Remote sensing is a very useful method of acquiring information to prepare a comprehensive water resource management plan, and to monitor the progress of the plan.

It is visualized that some of the immediate benefits from remote sensing data will include: 1) improved inventories and analyses of marine and freshwater resources, 2) new techniques for monitoring the movement of selected bodies of water, 3) comprehensive evaluation of the water community and associated terrestrial resources, 4) timely detection of pollution sources and their effects upon plants in the near-surface environment, and 5) accurate data that can be used to develop water resources for the greatest benefit with the least damage to the environment.

Remote sensing technology may provide very important data in the exploration, development, conservation, and disposal of water resources in this decade. It surely will help in man's attempt to increase food production from the sea, and to solve water resource use and management problems the world over.

Findings

The remote sensing study conducted here has demonstrated the potential of selected techniques for data collection in the marine and freshwater environment as man attempts to conserve the water resource and utilize the tremendous resources of the oceans and coastal areas. It has shown that current remote sensing techniques provide only a small portion of the total information that can be obtained in this way.

The techniques used included taking multiband aerial photography and underwater photography, collecting water specimens, on-site observation, and image interpretation. Multiband black and white photography was taken and compared with color and color infrared photographs of the same area and the results are tabulated in Table I. It was found that many of the features of interest were detectable on the black and white imagery, but that it was possible to confuse important conditions with other features that were not of interest. The color imagery eliminated much of the ambiguity and was considerably easier to use than the multiple black and white images, but it suffered from loss of color fidelity when used at high altitudes.

Color infrared proved to be the most useful material for evaluating floating vegetation in both freshwater and marine areas including locating kelp beds, and identifying kelp types. Where water clarity permitted, this material also provided considerable underwater information. There is an advantage to using color infrared film over color film at high altitudes because a haze-cutting filter is used with this film providing better color rendition (contrast). Multiband images that were projected to form a color image were not found to be as useful as conventional color film for these investigations and were much more difficult to handle.

TABLE I

PHOTOGRAPHIC SPECIFICATIONS

Problem	Best Film-Filter Combination[a] (Wratten Filter)	Best Black and White Film-Filter Combination(s)[a]	Smallest Scale Used Successfully	Notes
Water Movement	Color-1A	Pan 25A Pan 58 Pan 90	1/60,000	Some water impurities caused confusion
Detecting Pollutants-General	Color-1A	Pan 25A Pan 90	1/60,000	Color is significantly better than black and white
Oil Pollution	Pan 47B Ortho 18A	Pan 47B Ortho 18A	1/8,000	Overcast day appeared to be best
Inventory of Kelp	Color Infrared-12	Infrared-89B	1/24,000	Sun glare caused problems

[a] Numbers designate Wratten filters. Partial list of suitable films:

Color: Kodak Aero Neg. (MS), Ektachrome Aero, Ektachrome X, Kodachrome II, and Anscochrome.

Color Infrared: Kodak Ektachrome Infrared Aero.

Pan (Panchromatic): Kodak Plus-X Aerographic, Double-X Aerographic, and Tri-X Aerecon.

Ortho: Kodak Royal Ortho, Commercial.

Infrared: Kodak Infrared Aerographic.

Water pollution sources could be detected if 1) the water were discolored, or 2) visible deposits or floating debris were left on the shoreline, or 3) if plants or animals were affected in a way that could by detected on the aerial image. It was not possible to identify either the pollution material or the concentration level, although in many cases the source could be readily located, thus leading to identification. Algae types could not be identified on color aerial photography. As reported by astronauts observing reefs and shoals in the ocean from their vantage point of 100 to 150 miles up, more information on location of underwater sediment beds is obtainable from high altitude photography than from low altitude photos.

THE USE OF REMOTE SENSING IN WATER MOVEMENT STUDIES

The use of sea dye for monitoring movement of currents and tributary streams in bays was investigated in two areas.

One was Humboldt Bay; the other San Diego Bay. In Humboldt Bay there is a low shoreline utilization by cities and industry; the Bay is shallow at low tide leaving a large area of exposed mud flats; the north end of the Bay is not navigable by large ocean-going vessels, and there is a high flushing action by tributary streams and tides. Pollution levels have been high in Humboldt Bay in spite of the high flushing action and have forced the closing of some areas where oyster production would otherwise be undertaken.

San Diego Bay was selected to provide a contrast There is high shoreline utilization; the Bay is essentially a deep water Bay with relatively small areas of mud flats at low tide; it is navigable by large naval vessels, and there is almost no flushing action by tributary streams and only moderate flushing by tides. The pollution levels are quite high in San Diego Bay because of the activities of industries, municipalities, and the disposal of untreated sewage by naval vessels moored in the Bay.

Sea dye releases were made in both bays and aerial photography was taken during these investigations to evaluate the current movements in the bays and the possible dispersion of pollution sources. Figure 1 illustrates the conditions in Humboldt Bay.

Humboldt Bay

Tributary streams around the east side of Humboldt Bay were highly polluted because of several factors. These included 1) the dairy industry's practice of washing waste from their barns into the tributary streams, 2) the logging industry not adequately protecting the watershed from erosion and clogging of streams by slash, leaves, and debris, 3) the inefficient septic tanks located in homes on the watershed, and 4) sewage treatment plants that were not properly treating effluents. Therefore, it was desired to monitor the movement of tributary streams into Humboldt Bay and determine if they were contributing to pollution of the oyster beds.

Color aerial photos are significant sources of information in monitoring pollution sources relative to their dispersal and areas affected.

Rhodamine B sea dye was used in the Humboldt Bay investigations. This dye has been used for monitoring water movement for several years, and has proven to be an excellent material for such studies. It has a long life, is nontoxic, and can be evaluated by fluorometer for concentration information. The dye is balanced in specific gravity for sea water studies.

To ensure adequate mixing after release, the dye was diluted 10 to 1 with water obtained from the Bay near the release point. Starting at a morning high tide, 30 gallons of diluted dye were released in about one pint increments at one-minute intervals during the ebbtide into a tributary stream known to be polluted. (See Figure 2.) At the same time, milk bottle caps were released to evaluate the suitability of using such devices for monitoring water movement. Wind immediately forced the bottle caps to the nearby shore and eliminated them as tracers of water movement. Aerial photographs were taken using multiband black and white color films periodically during the dye release to record the dye movement.

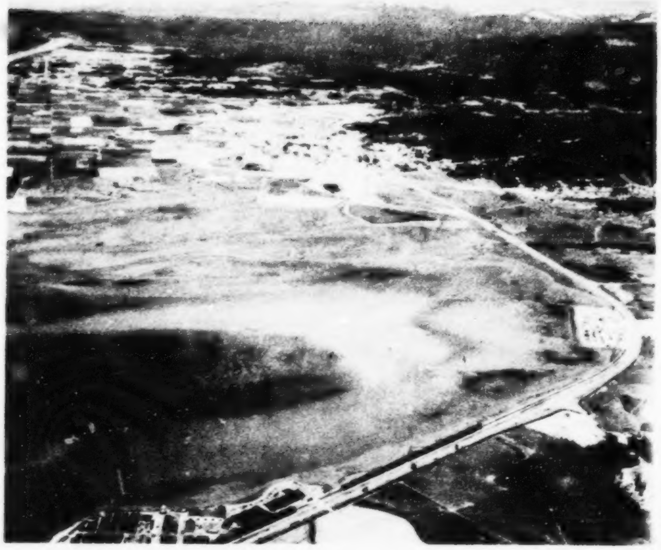

Figure 1. *Panchromatic 25A photo of NE side of Humboldt Bay. Note low shoreline utilization and shallow water at low tide.*

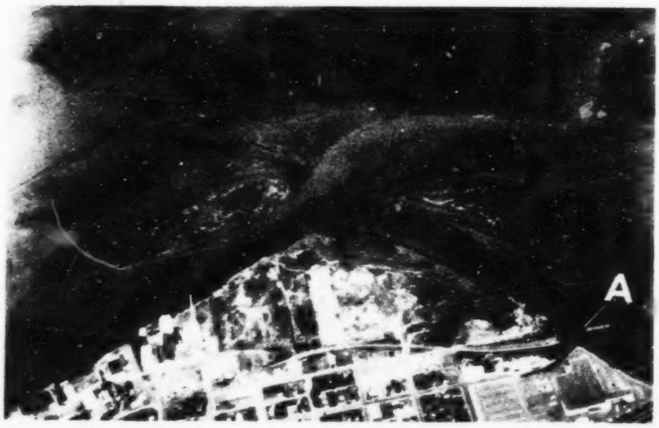

Figure 2. *Panchromatic 58 (green) photo of red dye in Eureka Slough in Humboldt Bay showing current flow at ebb tide. Rhodamine B (red) dye release at A was at railroad bridge.*

The dye experiment was designed to follow the flow of polluted tributary streams during a tidal excursion to evaluate their contribution to pollution in the oyster beds. The photographs readily demonstrated that the dyed water from these channels did not run directly out to sea during ebbtide as had been thought by some observers, but instead moved outward toward the ocean only to be dispersed and returned to many parts of the Bay during high tide.

San Diego Bay

In a study performed in San Diego Bay, sea dye was used to determine tidal flushing action relative to pollution problems in the Bay. Rhodamine WT was released by the FWPCA continuously from a single point to simulate a pollution source in the Bay such as a large naval vessel tied up at a pier with 2,000 to 3,000 men on board. Two small boats were used to record dye concentration in the Bay waters by on-board fluorometers. These boats were operated over scheduled courses during the dye study. The photo aircraft took aerial photos of the Bay periodically to detect dyed water and to guide the boats to areas outside their regular routes where suspected dye concentrations were observed. Figure 3 is a view of the central portion of San Diego Bay taken on panchromatic film with a Wratten 25A filter which reveals the intensive development of shoreline, the presence of large naval vessels indicating deep water, potential pollution sources in the metropolitan and industrial areas, and the apparent absence of tributary streams to provide flushing action. It was found that dye in very low concentrations, as small as 3 to 5 parts per billion, was visible on aerial photography.

In establishing specifications for aerial photography of diluted dye, it was not expected that dye in very low concentrations would be detectable on the aerial photographs. However, it was found that when the water clarity permitted visibility to a depth of 8 to 10 feet, very low concentrations of dye were visible because of the integrating effect when looking through a column of dye 8 to 10 feet long. It is obvious that if a small quantity of water such as that collected in a test tube were observed, it would not be possible to detect clear water from dyed water in low concentrations. However, if one looked down the length of a tube of dyed water, 8 to 10 feet long, it would be possible to determine the difference between clear and dyed water because of the integrating effect of thousands of molecules of dye that would be visible in such a column of water. In color aerial photography, dye is not as detectable in turbid water as it is in clear water for the following two reasons:

1. A masking effect of impurities prevents seeing into a column of water to a sufficient depth to take advantage of the principle just described.
2. The colors of the impurities may tend to be similar in color to the dye, reducing its detectability.

Table II lists the findings of dye detectability in the San Diego study.

TABLE II

DETECTABILITY OF RHODAMINE WT DYE AND POLLUTANTS AT SAN DIEGO

Film-Filter	Relative Tone of Dye and Detectability on Positive Image	Relative Tone of Other Impurities Observed on Positive Image
Pan 12 (yellow)	Light-fair	Light
Pan 25A (red)	Light-good	Light
Pan 47B (blue)	Undetectable	Light
Pan 58 (green)	Dark-good	Light
Pan 72B (brownish red)	Light-fair	Variable
Pan 90 (gold)	Very light-good	Variable
Pan 99 (sea green)	Undetectable	Variable
Infrared 89B	Not recommended	Not Recommended
Color-1A (haze)	Red-good	Variable
Color Infrared 12	Yellow-good	Variable

THE USE OF REMOTE SENSING IN POLLUTION CONTROL

Remote sensing has been used for detecting pollution sources for many years. An outfall could be detected from discoloration of the water. If the channel water was already heavily laden with suspended particles the effluent outfall may not have been detectable for more than a few feet from its source.

Classically, water pollution has been defined as anything that degrades water, making it toxic to plants and animals or unfit for industrial use. The term pollution was used primarily to describe waste materials released by man. However, managers of water resources have come to realize that any system for classifying water pollution must also include listings of materials occurring naturally such as silt, sand, rocks, debris, algae, and microscopic organisms. Any of these materials combined with man-caused impurities increases the burden that must be carried by a body of water. If a stream or lake is already laden with naturally occurring organisms and inorganic materials, the addition of any substance, which in itself would have few long-lasting effects, may drastically choke an already full stream.

In remote sensing studies for water resource management, it is necessary to take into account the fact that water managers must have accurate information on the total load of impurities that a body of water is carrying regardless of the source of the impurities.

In flights around California, observation of various bodies of water revealed that it was possible to detect the point where water quality changed, such as at the confluence of two streams or from sewage outfalls. Figure 4 is a photo of the mouth of the Russian River in Northern California where springtime runoff has caused high levels of fine sediments to be washed into the Russian River and tributary streams, and has discolored the water. Note how the freshwater from the river floats on top of the salt water, and note how the sea water is visible where offshore rocks have distrubed the current pattern.

In searching for sources of silt that could account for the high level of turbidity in the Russian River, the photo in Figure 5 was taken showing the confluence of Austin Creek and the Russian River. It is possible to see on this photo how the relatively clear water of Austin Creek disappears into the heavily silt-loaded Russian River which carries water from areas of very unstable soil in Sonoma and Mendocino counties. For navigation purposes, it is necessary to detect sandbars in channels. Sandbars may shift from season to season depending on current velocity, channel configuration, and silt-load conditions. If a sandbar is covered with perennial vegetation, it can be assumed that the sandbar is stable and, while a hazard to navigation, it will remain stationary. Immediately to the right of the concrete highway bridge on Austin Creek a sandbar can be seen that is classified as unstable because of the lack of vegetation on it. It is more difficult to determine the presence or absence of vegetation on conventional color photos than on color infrared photos.

Davis Oil Pollution Study

Oil products have caused water pollution in many areas of the world. These pollutants affect shorelines, docks, ship hulls, fish, birds, animal life, vegetation, and swimmers. Some of the sources of these pollutants are readily visible and can be corrected; others are not so easily detected and require extensive investigation to locate the cause of the oil slick. Often natural seepage is responsible for oil deposits on land or in water, and correction is not easy — as exemplified by the recent Santa Barbara channel oil pollution problem. Industrial activities and shipping are probably two of the prime contributors to oil pollution in the United States.

Many users of the nation's waterways consider water an excellent carrier of waste products and therefore a way to get rid of unwanted oil. Others assume that a little leakage during transport operations will be carried away and will not cause lasting effects on the local community. Oil, however, is generally a persistent pollutant on water and is difficult to remove once it accumulates in a bay or harbor.

It is desirable to detect causes of oil pollution quickly and establish responsibility for the action so that corrective measures can be made. A study of remote sensing techniques that could be used to locate sources of oil pollution along a freshwater or marine waterway was undertaken with the following results.

At the Davis campus of the University of California, multiband imagery of a water tank array was taken from the top of a 150-foot tower to simulate aerial photography. In a series of tests, numerous oils were dropped on the water of these tanks in increasing quantities, and multiband photographs were obtained after each oil addition. These photographs were then compared to determine the relative usefulness of each film-filter combination in identifying the oil slick.

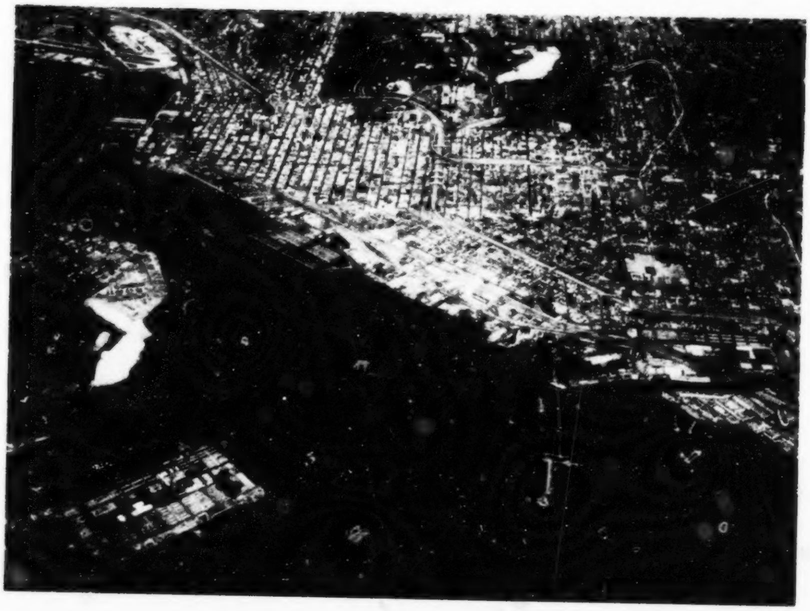

Figure 3. *Panchromatic 25A (red) photo of San Diego Bay. Note high shoreline utilization and deep water anchorage.*

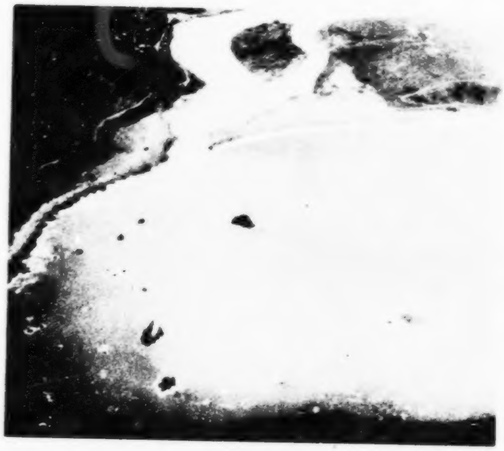

Figure 4. *Panchromatic 25A (red) photo of mouth of Russian River showing silt from spring run-off.*

It was found that some spectral bands permitted the detection of oil slicks and other bands did not. The red, yellow and green bands most frequently used for aerial survey work were the least useful for detecting oil slicks; for oil detection the bands in the blue and ultraviolet parts of the spectrum were the most useful.

Generally, the blue parts of the spectrum are filtered out in aerial photography because of the magnitude of scattering of light by intervening haze in that part of the photographic spectrum. While it is not wise to generalize with the limited number of conditions investigated in this study, aerial imagery in the blue part of the spectrum has been obtained of oil pollution areas in San Diego Bay. Detectability was good for that part of the spectrum but not for others. Color photographs did not permit useful detection in either the aerial photographs at San Diego or tower photographs at Davis where very small amounts of oil were released. However, in extensive ship oil pollution areas in the San Diego Bay study, color photographs were found by the author to be useful.

Limited experiments at Davis have demonstrated that oil slicks on water can be detected using black and white imagery, depending upon the spectral band exposed. The best detection occurred in the blue and ultraviolet parts of the spectrum; the poorest occurred in the infrared, red, yellow, green, and in combinations of those spectral regions. Color photographs were very limited in their ability to record thin oil slicks. Table III presents the interpretation data developed from the Davis investigation.

The "rainbow" associated with oil slicks is a function of the varying emulsion thickness of oils on water. The highly-refined motor oil used in the Davis test was the least detectable and had the least obvious rainbow. This factor is probably due to the uniform emulsion thickness that occurs when this product is put on water.

Additional investigations are suggested by this study. Future studies should consider the following factors:

1. Is there an optimum sky condition for detecting each of the oils of interest?

2. Is there an optimum combination of sun angle, camera angle, and sky condition?

3. What effects do air and water temperatures have on detectability?

4. What effect does the presence of other impurities in the water have upon detectability?

5. Is there a difference in detectability of oils on fresh and salt water?

TABLE III

RELATIVE USEFULNESS OF SPECTRAL BANDS FOR OIL DETECTION

Wave-length mμ	Filters	So. Reg. Crude	Zaca Crude	Inglewood Crude	Cracker Cutter	Diesel	30 Weight Motor Oil	Weed Oil	Boiled Linseed Oil	All Oils Mixed
500-650	12	None	None	None	None	None	None	None	None	None
320-390	18A	Good	Good	Good	Good	Good	None	Good	Poor	Poor
590-650	25A	None	None	None	None	None	None	None	None	None
350-450	35	Good	Good	Fair	Fair	Fair	None	Fair	Poor	Poor
395-470	47B	Good	Good	Poor	Fair	Good	None	Good	Poor	Poor
495-580	58	Poor	Fair	None	None	Fair	None	Fair	None	None
470-500	75	Fair	Fair	None	None	Poor	None	Fair	None	None
700-950	IR 89B	None	None	None	None	None	None	None	None	None
350-650	Open	Poor	Poor	None	None	Poor	None	Poor	None	None
350-650	Color	Fair	Good	Poor	Fair	Fair	Poor	Good	Poor	Poor
500-900	Ek IR	None	None	None	None	None	None	None	None	None

Lake Tahoe Study

The vast quantities of sediment that tributary streams are carrying into the waters of Lake Tahoe are important factors in the contamination and degradation of the lake waters. These sediments result from erosion within the drainage basin; this erosion is closely connected with the amount of construction work being undertaken.

Deposits of sand, silt, and clay have collected along the margin of the lake at the stream outlets which drain into the lake. (See Figure 6.) In many cases these deposits are accumulating today. The deposits consist of fine to medium grain sand, silt, clay, and minor amounts of gravel, most of which is well rounded, (Burnett 1967).

Color photography taken of the lake reveals details of sediment beds, a form of pollution, at Southshore Lake Tahoe. Depth measurements were taken at several locations in this area. Changes in blue color on color film coincide with increases in depth — the light blue area is shallow and the dark blue area is deep. Underwater vegetation is visible in many areas as dark lines running out from the shore. If only color photos are available one might think that the vegetation is floating. However, on the color infrared photo submerged vegetation is not red or pink as is the terrestrial vegetation and thus it is underwater. To assure adequate depth penetration it was necessary to overexpose the color infrared by about one stop from the exposure used for forest photos.

Humboldt Bay Study

Several pollution sources were photographed at Humboldt Bay during the experiment on tracing of tributary streams by sea dye. Figure 7 shows an unknown dark liquid being discharged from a sawmill and Figure 8 shows runoff from sprinkler systems on log-storage piles in the Bay. Photos such as these can be used to locate offenders and often to enforce the regulation against pollution without court action.

Aerial views in color infrared provide pollution investigators with very useful information and up-to-date photo maps of their area of responsibility.

San Diego Bay Study

Oil pollution from naval ships moored in San Diego Bay was detected during the sea dye experiment in September 1967. The best oil detection occurred on overcast days.

As seen in Figure 9, oil pollution was detected using panchromatic film with a Wratten 47B (blue) filter from the dredge visible in that photo. No oil was visible either to the flight crew or on color photos of the area taken to monitor dye dispersion. Subsequent photo interpretation showed the suspected oil slick, its source, and the plume as it drifted with the flood tide. Experience has shown that for photographic applications a thin oil slick is detectable only in the blue part of the spectrum. An oil slick is visible when its reflection of the sky conditions result in its being light in tone on a clear day on the blue filtered image. On an overcast day oil, which reflects white clouds, is easily detectable against blue-green background water. On clear days however, there was very little contrast between blue-green water and oil, which was reflecting the blue sky. The fact that oil slicks are more detectable on overcast days might lead one to believe erroneously that ships only dump oil on overcast days.

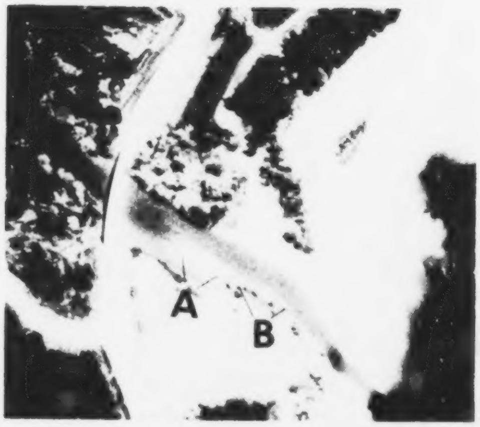

Figure 5. *Panchromatic 25A (red) photo of confluence of Russian River with Austin Creek. Unstable sand bar is at A, stable sand bar is at B.*

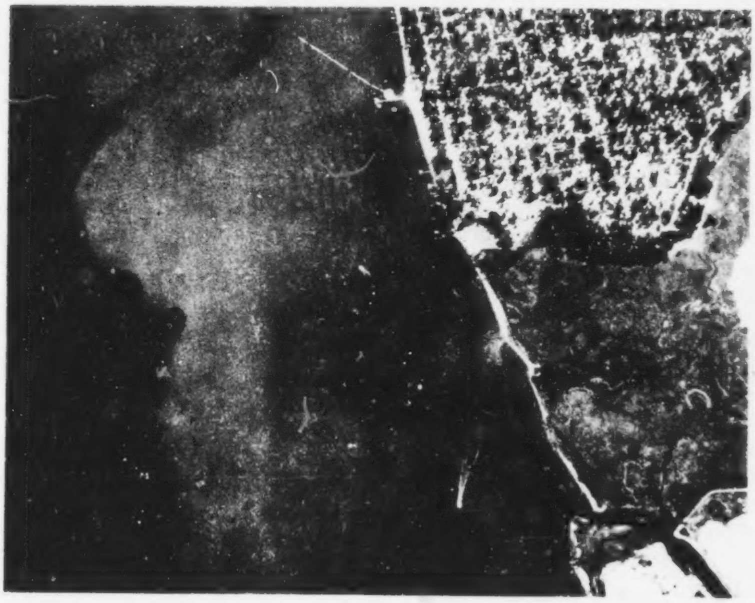

Figure 6. *Panchromatic photo (no filter) of Southshore Lake Tahoe showing underwater sediment bed. See text for explanation.*

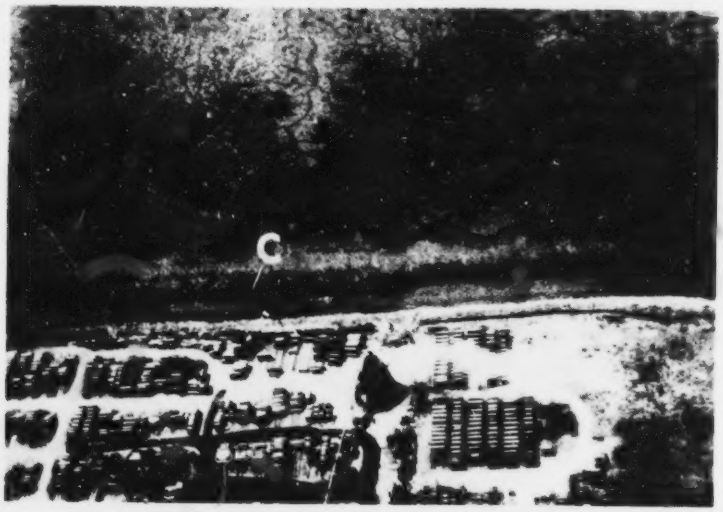

Figure 7. *Panchromatic 58 (green) photo of water pollution source in Humboldt Bay at C.*

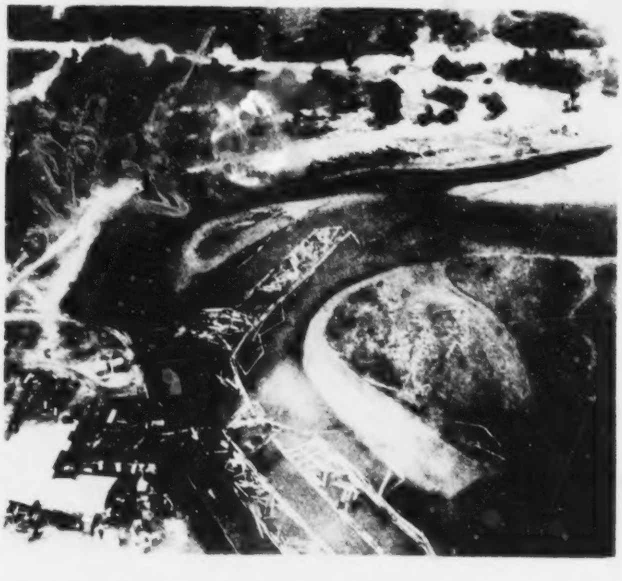

Figure 8. *Panchromatic 25A (red) photo of water pollution in Mad River Slough, Humboldt Bay.*

Figure 9. *Panchromatic 47B (blue) photo of oil pollution in San Diego Bay at A.*

The studies presented here were not designed to solve any particular problem but instead were intended to show the scientists and managers concerned with the water resource problems of the nation that remote sensing offers tremendous potential as a data source for evaluating the physical and environmental conditions that dominate an area.

Often the true conditions of the scene and its components cannot be determined by image interpretation alone, when these interpretations are made in an office many miles away with only viewing aids and references available. It therefore behooves the interpreter to visit the area being studied as frequently as necessary, studying the conditions with imagery in hand, until he is confident that his interpretations bear as close a relationship as possible to the true ground conditions. He is then free to proceed to other areas where similar conditions exist or to imagery acquired of the same scene taken at different times, confident that his interpretations will be accurate and meaningful.

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SEDIMENT – WATER NUTRIENT INTERCHANGE

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INTRODUCTION

Lake sediments have been recognized as a possible source of nutrients for undesirable algae and aquatic plant growth. The physical, chemical, and biological processes at the mud-water interface have long been considered as significant factors in the release or removal of nutrients. These processes are closely interrelated and probably are much more complicated than originally described by early investigators.

In order to eliminate unwanted algal and aquatic plant growths from lakes, the National Eutrophication Research Program of the Federal Water Pollution Control Administration has been conducting research to learn what happens at the sediment-water interface in any given ecosystem so that methods may be devised to reduce or eliminate release of nutrients. The purpose of this paper is to discuss some of the results of this program and the problems encountered.

NUTRIENT INTERCHANGE PROCESSES

Mortimer (1941a, 1941b) reported that iron, ammonia, silicate, phosphate, alkalinity, conductivity, and substances reducing permanganate increased in concentration in oxygen-deficient water over sediments which became reduced ($E_h < 0.2$ volt). Hayes and Anthony (1958) also observed liberation of inorganic phosphate from bottom muds in stratified lakes when the water became low in oxygen. Uptake of dissolved inorganic phosphate by sediment has been considered to take place under aerobic conditions (Holden 1958, Jitts 1959, and Olsen 1964).

Release or uptake of phosphate in sediments is pH dependent. It has been shown that oxidized mud from unproductive, medium productive, productive, and acid bog lakes will not adsorb phosphate at pH values greater than 7 nor less than about 4.5 (MacPherson et al. 1958). The adsorption properties of Chesapeake Bay and Roanoke River sediments, bentonite, Fuller's earth, and powdered pyrex glass toward inorganic phosphate, iron (III), and other ions were studied under varying conditions of temperature, salinity, pH, and contact time by Carritt and Goodgal (1954). They concluded that phosphate could be released to a soluble form from a sorption complex in the sediments when exposed to a higher pH. Ferric phosphate ($FePO_4$) is much more soluble at higher pH values (Morgan and Stumm 1964), therefore, phosphate could be released by dissolution processes under certain lake conditions. In eutrophic lakes the pH of the water commonly reaches 9.5 or 10 during heavy algal growth.

Since there are different types of sediments, analogous to different types of soil, it is very likely that the cycling process of phosphorus varies among lakes. Eventually lake sediment — water equilibration systems may be categorized by several main types similar to those suggested by Golterman (1969): Fe-S-P, Ca-CO_3-P, Fe-humus-P, and Al-Si-humus systems. An understanding of the chemistry of each of these systems would be useful for developing techniques for eliminating sediment nutrient release.

Physical processes in lakes cause nutrients in the sediments to interchange. These processes include the effect of natural flow of water over the sediments or by stirring action of fish, boats, or seiches; movement of sediments by wind action upon the overlying water which may resuspend the upper layer of sediment; effect of eddy diffusion; disturbance of the solid phase by gas formation and release from the mud; diffusional processes; and hydraulic head.

The effect of wind on resuspension of sediments depends upon the size and depth of the lake as well as the physical properties of the sediment. In shallow lakes the effect may be very significant whereas in deep lakes resuspension may be negligible.

The biological processes taking place in sediments are very significant in interchange. Benthic organisms physically alter the sediment by their movement and life processes. From 7,000 to 17,000 benthic organisms per square meter have been found in Upper Klamath Lake sediments (Bond et al. 1967). These were primarily oligochaetes and leaches. It is interesting to contemplate the stirring and nutrient changes by these organisms upon the sediments. Some types of algae, such as Oscillatoria form on the surface of the sediment and collect gases to such an extent that the algae and some of the sediment floats to the surface of the lake.

Bacterial activity, which is probably one of the most important factors in interchange in some sediments, depends upon temperature, nature and amount of the organic matter, chemical and physical composition of the sediments, pH, E_h, and other variables.

Microorganisms have been reported to solubilize "insoluble" inorganic phosphates and this process may have been overlooked by investigators in the field. Wood (1965) refers to ZoBell's original statement of conversion of insoluble tricalcium phosphate into cell proteins and phospholipids by direct assimilation. Wood (1965), by use of a calcium or ferric phosphate medium with asparagin as a carbon source, was able to grow bacteria at pH 8.5. These bacteria, which had been cultured on the ferric phosphate medium for twelve months were able to grow more actively on the calcium phosphate medium than on the nutrient agar and continued to grow on the ferric phosphate medium. This general idea is repeated in a more recent book (Wood 1967) which also states that colloidal phosphate from clay-loams can be consumed.

According to Alexander (1961), species of Pseudomonas, Mycobacterium, Micrococcus, Flavobacterium, Penicillium, Sclerotium, and Aspergillus grow in media with $Ca_3(PO_4)_2$, apatite, or other insoluble materials as sole phosphate sources. In the case of ferric phosphate solubilization, one proposed mechanism suggests that bacteria liberate hydrogen sulfide which reacts with ferric phosphate to yield ferrous sulfide, thus releasing phosphate.

One general method for lake restoration suggests that the phosphorus cycle be interrupted by precipitation of the phosphate by a metal ion such as aluminum which will form an insoluble precipitate that will settle to the bottom and be incorporated in the sediment. If the microorganisms will dissolve the water "insoluble" phosphates, lake restoration by this means may be only temporary. Initial work by Dr. R. Pacha at Oregon State University confirms that lake sediment bacteria from Upper Klamath Lake, Oregon, dissolve calcium phosphate. A program for the study of dissolution of other insoluble phosphates by microorganisms found in Upper Klamath Lake is in progress. Incidentally, Pseudomonas and Flavobacterium were predominant bacteria found in the Upper Klamath Lake sediments during the winter and early spring.

Bacteria, of course, are responsible for the formation of gases and other chemical changes which alter the pH and E_h of sediments.

FIELD DETERMINATION OF INTERCHANGE

The determination of the occurrence and extent of interchange in an ecosystem is not measured easily at the present time. Although laboratory tests can be made, the exact ecological conditions of the lake such as biological factors, radiant energy, meteorological and other physical conditions, etc. cannot be reproduced in the laboratory. One of the objectives of the Sediment-Water Interchange Section of the National Eutrophication Research Program since August 1967 has been to devise methods for measurement of interchange of nutrients initially working with the highly eutrophic Upper Klamath and Agency Lakes in South Central Oregon.

A field experiment was initiated which consisted of installations of four polyethylene (10 mil thickness) pools 10 ft x 10 ft x 15 ft deep placed in a wood framework in a location which appeared to be representative of the lake. Two pools were bottomless so that the lake water was exposed to the sediment, whereas the other two pools had plastic bottoms so that the lake water was not exposed to the sediment. The pools were installed in November 1967 near the end of the Aphanizomenon bloom. The physical, chemical, and biological variables of the water and sediment in the pools were observed from the surface to the bottom at least monthly and compared with the surrounding lake water for about seven months, until cracks and holes developed in the plastic.

However, some useful information was derived from this field experiment. It was found that under the ice, which formed in early December and remained until late February, the oxygen content averaged 2.3 mg 0/ℓ higher in the plastic-bottom pools than in the sediment-bottom pools and 5.8 mg 0/ℓ higher than in the lake. In the spring, floating Oscillatoria and lake sediment did not appear on the water surface in the plastic-bottom pools. Although a small hole had developed in the one remaining good plastic-bottom pool, the Aphanizomenon growth was not nearly as intense in this pool as in the sediment-bottom pools.

Our experiment showed that although testing interchange under field conditions would be ideal, it is experimentally difficult because of the length of time for the experiment, the expense of installation and observation of a set of pools, and the difficulty in testing many lakes by this method. There are other problems that should be pointed out. The pools were rather large and supposedly designed to overcome wall effects but in early spring a mat of the diatom, Gomphonema, formed on the plastic walls and reduced the soluble silica in the water to half the level that occurred in the lake water (10 mg SiO_2/ℓ) at that time. It is obvious that deep lakes cannot be tested by this technique. Small circular-shaped pools, two feet in diameter, were tested but were found to be unsatisfactory.

Observations of physical and chemical changes in the lakes were made in an effort to determine whether interchange could be detected as described by Mortimer (1941a, 1941b). Under the ice, which remained continuously over most of the lake about two and one-half months, oxygen in the water became low (less than 2 mg/mℓ) from the surface to the bottom. Since wind action was non-existent, it was thought that the water near the bottom should increase in phosphorus and nitrogen content. This did not prove to be the case. Iron (II) was not detected except for a very faint test at one location.

In the spring and autumn, large masses of Oscillatoria and attached sediment up to 4 to 6 in. in thickness were observed floating on the surface in several of the bays. In Howard Bay when this action occurred in September the conductivity increased to 185 micromhos/cm in contrast to 122 in the main portion of the lake. Ammonia-nitrogen and total phosphorus also increased to 1.2 mg N/ℓ and 1.0 mg P/ℓ, respectively, compared with about 0.1 mg N/ℓ and 0.2 mg P/ℓ in the main part of the lake. Oxygen, also, became very low (less than 3.0 mg/ℓ) and small fish, 4 to 8 cm long, died. This is a definite indication of sediment-water interchange, but quantitative evaluation of this effect upon the whole lake system would be difficult.

LABORATORY INTERCHANGE TESTS

Since field experiments of the type described are difficult to perform, new types of field tests or laboratory tests upon sediments will need to be devised. Although several investigators have tested nutrient interchange of sediments in the laboratory under aerobic and anaerobic conditions, at present there are insufficient analytical procedures for laboratory evaluation of nutrient availability and interchange. A trend has been to apply soil tests and biochemical procedures. These may or may not be applicable and will require evaluation and possible modification. It should be emphasized that analysis of sediments for total phosphorus, nitrogen, carbon, etc. is of no real value unless the "availability" of that element to the interface or overlying water can be determined. This will depend upon the various conditions of pH, E_h, oxygen content, etc. that develop in the lake water and sediment in any particular ecosystem.

NUTRIENTS IN SEDIMENT INTERSTITIAL WATER

In an effort to determine nutrient availability in sediments, it was decided to determine the concentration of the soluble nutrients in interstitial water from Upper Klamath and Agency Lake sediments. If measurable concentrations of nutrients were present, these would be released to the overlying water by the various physical and biological processes previously described. Harriss (1967) has stated, "The composition of interstitial waters from river and lake sediments is controlled by a complex interaction of the groundwater recharge system, mineralogical dissolution and precipitation reactions, biological activity, and the degree of physical interaction between the sediment and overlying water." He studied the soluble silica and chloride concentration in interstitial water from freshwater sediments; chloride concentration changes were useful for studying diffusional processes in sediments. Several investigators have measured soluble constituents in interstitial (pore) water for the purpose of studying mineral-water equilibria (Sutherland et al. 1966) and mineral transformations (Siever 1965). Gorham (1961) suggests that ions would diffuse from the interstitial water to the overlying water, particularly during stormy periods. However, no one apparently has analyzed interstitial water from various lakes for the purpose of evaluation of the soluble nutrients present which might be readily available to the overlying water. Sullivan (1967) has shown that orthophosphate increased in the sediment interstitial water from Lake Bloomington during stratification with a decrease following turnover.

The presence of soluble nutrients in sediments was tested in samples from various eutrophic lakes in the western part of the United States. The samples were centrifuged at 13,000 rpm in 250-mℓ polycarbonate bottles in a refrigerated centrifuge (4°C) and the separated interstitial water filtered through a 0.45-micron membrane filter. The interstitial water from these sediments contained relatively high concentrations of soluble phosphorus and ammonia; from 0.08 to 10.5 mg P/ℓ and 1.5 to 86 mg N/ℓ (see Table I). Very little phosphorus ($<$0.01 mg P/ℓ) was present in the interstitial water from the oligotrophic lake (Woahink). Up to 17 mg P/ℓ and 140 mg N/ℓ have been found in Upper Klamath Lake sediment interstitial water.

The concentration of nutrients is higher than expected and any physical process which will disturb the sediments or resuspend them into the overlying water will cause nutrients to become available.

The change in nutrient concentration with depth in sediment cores is shown in Table II. Nitrogen, as ammonia, increases in each example cited, whereas phosphorus varies. This type of information would be useful when lakes are to be restored by dredging of the sediments. At the Howard Bay location (Table II) the sediment below the surface contained more soluble nutrient than at the surface.

LABORATORY EQUILIBRIUM TESTS

Interchange tests in 6-in. diameter, 6-ft high glass columns with Upper Klamath Lake sediment and water under both anaerobic and aerobic conditions showed an increase of both phosphorus and nitrogen in the overlying water. Since the sediment was high in soluble interstitial nutrients, this could have been accomplished just by diffusion and by stirring from the gassing process. An unusually high concentration of nitrite (3.5 mg N/ℓ) developed in both the aerobic and anaerobic columns which persisted for several weeks. Nitrite was never found to be greater than 0.02 mg N/ℓ in the lake so that the laboratory conditions did not correspond to those in the lake.

Since the orthophosphate concentration decreases to very low values in lake water during periods of algal growth, the phosphorus equilibrium from a consideration of only chemical processes should be directed from the sediment to the water. Laboratory experiments have been carried out by growing Selenastrum in aquariums containing sediment in contact with lake water, and lake water only for the control to determine whether interchange takes place under these conditions.

TABLE I

NUTRIENT CONCENTRATION IN SEDIMENT INTERSTITIAL WATER FROM VARIOUS LAKES

Determination mg/ℓ	Upper Klamath Lake, Oregon Howard Bay	Upper Klamath Lake, Oregon Buck Island	Agency Lake, Oregon	Salton Sea, Calif.	Shagawa, Minn.	Sallie, Minn.	Herman, South Dakota	Clear Lake, Calif.	Woahink, Oregon
P-ortho	10.5	0.11	0.08	1.0	0.15	0.44	1.5	0.08	< 0.01
P-total soluble	10.5	0.27	0.18	1.3	0.36	0.55	1.5	0.10	
$N\text{-}NH_3$	86	9	4.4	16.5	3.5	1.7	3.0	0.39	
N-total Kjeldahl	86	11	5.7	16.9	5.2	3.9	5.2	1.8	
$N\text{-}NO_3$	(< 0.05)[a]	(<0.05)	0.02	0.12	<0.05	<0.05	0.08	0.07	
$N\text{-}NO_2$	(< 0.02)	(<0.02)	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	
C-total	141	37		183	28	79	209	23	

[a] Values obtained on sediments over several months.

TABLE II

NUTRIENT CONCENTRATION IN SEDIMENT INTERSTITIAL WATER FROM CORES[a]

	Depth, cm	0-30	30-60	60-90	90-120	120-150	150-165
Upper Klamath Lake (Buck Island, 9-24-68)	P-ortho	0.07	0.11	0.08	0.05	0.03	0.04
	P-total soluble	0.17	0.22	0.27	0.17	0.19	0.19
	N-NH$_3$	8.5	12.0	14.5	16.0	17.0	12.5
	N-total Kjeldahl	10.4	15.0	19.3	21.2	21.8	17.5
	N-NO$_3$	0.1	0.08	0.03	0.02	0.02	0.02
	SiO$_2$-soluble	49	49	49	43	41	42
	Alkalinity	82	97	115	131	127	
	Conductivity	191	231	266	298	306	211
Upper Klamath Lake (Howard Bay, 10-23-68)	P-ortho	8.5		16.5		17.5	
	P-total	8.5		17.0		18.2	
	N-NH$_3$	85		119		146	
	N-NO$_3$	< 0.01		< 0.01		< 0.01	
	N-NO$_2$	< 0.01		< 0.01		< 0.01	
	SiO$_2$-soluble	56		61		58	
	Conductivity	1022		1363		1659	

	Depth, cm	0-20	20-40	40-60
Salton Sea (11-13-68)	P-ortho	1.88	1.48	0.53
	N-NH$_3$	18.6	21.6	25.0
	N-NO$_3$	0.15	0.10	< 0.05
	N-NO$_2$	< 0.05	< 0.05	< 0.05
	Conductivity	61.3×10^3	87.6×10^3	117×10^3
Lake Herman (3-11-69)	P-ortho	0.84		2.4
	P-total soluble	1.0		2.6
	N-NH$_3$	5.75		14.0
	N-NO$_3$	0.05		
	N-NO$_2$	< 0.005		
	SiO$_2$-soluble	32.4		46.8

[a] Concentrations expressed in mg/ℓ; conductivity in micromhos/cm.

Selenastrum definitely grows better in aquariums containing sediment from various locations in Upper Klamath Lake than in aquariums without sediment, thus indicating some nutrient interchange. However, sediment from an oligotrophic lake (Woahink, Oregon) not only did not support algal growth but prevented growth. The reason for this is not known.

These experiments were carried out with or without mixing under aerobic conditions in the presence of radiant energy from fluorescent lights at room temperature.

Similar types of experiments were carried out in beakers so that the entire biomass in the water could be analyzed to determine the nutrient uptake by the biomass.

Algae grew well in distilled water in contact with Upper Klamath Lake sediments.

SEDIMENT TREATMENT PROCESSES

The ultimate purpose of the FWPCA research program in studying sediment-water nutrient interchange is to learn sufficiently about the processes involved to develop methods for the elimination of interchange.

Laboratory experiments to interrupt the phosphorus cycle by formation of insoluble phosphates of Al, Ca, Zr, La, Ti, etc. in sediments have been carried out in aquariums in the laboratory. Actually, much of the phosphate is probably adsorbed on the hydrous oxides. In Sweden pellets containing aluminum sulfate have been added to eutrophic lakes for restoration. It is too early to know how effective the treatment will be. Laboratory experiments with aluminum indicate that upon addition of 5 mg Al/ℓ and adjustment of the pH to about 7.0, algae grows readily. Additions of higher concentrations are now being tested. The aluminum hydroxide formed, along with adsorbed material, however, is very light and can be readily resuspended. If the pH of the water is high, solubilization could occur as well as mineralization by bacteria. Zirconium and lanthanum (or a didymium mixture) retard the algal growth more effectively than aluminum ions, but eventually an algal growth develops. It appears that this method of elimination might be only a temporary restoration measure and reapplication would be necessary. If the release of nutrients is sufficiently slow, they might be consumed by the biota in a manner so that undesirable algal blooms do not occur. The use of adsorbing solids (mineral or synthetic) of appropriate density and particle size to cover the sediments may be more effective. The use of nonadsorptive sand to cover the bottom with a thin layer would not be effective in many instances for several reasons. Some ooze-type sediments which are high in moisture content (> 90 percent) are low in density and the sand being of greater density would sink and not remain on the surface. Furthermore, the benthic organisms would mix the sand with the sediment so that the effect would be temporary.

Removal of sediments by dredging has been often suggested as a means for lake restoration. Questions arise pertaining to how much sediment must be removed and what is the available nutrient content of the exposed new sediment interface. It would be ideal to dredge to some layer of sediment which did not contain or release nutrients. Data from analysis of cores from Upper Klamath Lake and Salton Sea (Table II) show that the sediments contain appreciable nutrient down to the 6-ft level so that nutrient interchange could occur after dredging to that depth.

STABILITY OF SEDIMENTS

It is important in the study of nutrient availability in lake sediments to know whether sediments change appreciably upon standing under various conditions of storage. From the work on interstitial water of Upper Klamath Lake sediments, it was apparent that changes of soluble orthophosphate concentration in the sediments were taking place upon standing at 4°C. Freezing of soil has been reported to change phosphorus leachability (Bailey 1968). Therefore, several tests were made to observe the effect of standing and storage temperature upon sediments from various eutrophic lakes using the change in orthophosphate concentration in interstitial water as a measurement of the change. The data in Table III show that in Upper Klamath Lake sediments the following takes place: 1) The orthophosphate concentration in interstitial water decreases with time when stored at 4°C. 2) No soluble organic phosphorus compounds appear to form at 4°C. 3) An appreciable increase in soluble orthophosphate and organic phosphorus compounds result when stored at -16°C.

The changes which take place at 4°C are probably a result of bacterial activity. However, the increase in soluble phosphorus compounds from freezing the sediment cannot be readily explained.

Sediment from other eutrophic lakes (Salton Sea, Shagawa, Sallie, Herman) verify this effect of freezing (Table IV).

TABLE III

EFFECT OF TIME AND STORAGE TEMPERATURE UPON SOLUBLE PHOSPHORUS COMPOSITION OF INTERSTITIAL WATER IN UPPER KLAMATH LAKE, OREGON, SEDIMENTS

Dredge Samples taken 6/12/68 from Howard Bay
Storage Temperature, 4°C

Date of Analysis	P Ortho mg/ℓ	P Total Soluble mg/ℓ
6-18-68	2.9	3.1
6-25-68	2.2	-
7-01-68	1.3	-
7-03-68	0.2	0.29

Dredge Sample taken 8/14/68 from Howard Bay

Date of Analysis	Storage Temp. °C	P Ortho mg/ℓ	P Soluble Organic mg/ℓ	P Total Soluble mg/ℓ
8-15-68	(before storage)	9.0	0	9.0
8-28-68	4	5.7	0.2	5.9
9-13-68	4	4.9	0.1	5.0
8-28-68	-16	12.3	4.0	16.3
9-13-68	-16	15.0	6.5	21.5

TABLE IV

EFFECT OF FREEZING AND STORAGE TIME UPON LAKE SEDIMENTS AS MEASURED BY SOLUBLE CONSTITUENTS IN INTERSTITIAL WATER

Sample Location	Sample Identification	Date of Analysis	P Ortho mg P/ℓ	P Soluble Organic mg P/ℓ	P Total Soluble mg P/ℓ	N-NH3 mg N/ℓ	Total Kjeldahl N mg N/ℓ	Silicon Soluble mg SiO_2/ℓ	pH	Conductivity micromhos/cm
Salton Sea, California	1	[a]BF 11-27-68	0.95	0.30	1.25	14.8	15.3	36.5	8.1	47.0×10^3
		AF 12-30-68	1.65	7.35	9.0	13.8	22.5	29.3	8.0	52.3×10^3
	2	BF 11-27-68	0.48	0.02	0.50	12.7	13.2	32.5	8.1	41.4×10^3
		AF 12-30-68	0.90	11.1	12.0	12.2	21.8	24.0	8.0	47.2×10^3
Shagawa, Minnesota		BF 11-27-68	0.15	0.21	0.36	3.5	5.2	11.5	8.0	110
		AF 12-30-68	0.50	9.9	10.4	5.3	21.8	11.7	7.5	159
Lake Sallie, Minnesota		BF 12-27-68	0.40	0.15	0.55				7.6	522
		AF 2-11-69	1.3	5.4	6.7				7.6	627
		AF 3-10-69	1.7	4.6	6.3					678
Lake Herman, S. Dakota	1	BF 3-17-69	1.52	0	1.52	3.0	5.2	30.9	8.3	1446
		AF 4-10-69	0.40	0.38	0.78				8.0	1440
		AF 4-29-69	1.02	0.58	1.60	3.8	14.5	36.0	8.4	1438
	2	BF 3-17-69	2.24			3.0	5.4	36.0	8.3	1446
		AF 4-10-69	0.50	0.32	0.82				8.0	1466
		AF 4-29-69	1.34	0.16	1.50	2.0	11.5	40.0	8.1	1522

[a] BF, Before freezing
AF, After freezing and thawing

Thus, sediment samples must be analyzed as soon as possible after collection if a true evaluation of soluble orthophosphate and nutrient availability is to be determined.

This also raises the question whether freezing of sediments around the shallow edges of lakes might not cause similar solubilization of nutrients upon thawing in the spring, thus making available nutrients for algae and aquatic plants.

EFFECT OF STERILIZATION OF SEDIMENTS WITH COBALT IRRADIATION

It is necessary to separate chemical and biological processes in laboratory interchange experiments as well as in microbiological studies on sediments. Attempts to sterilize Upper Klamath Lake sediments with cobalt irradiation were made in cooperation with Dr. R. Pacha, Microbiology Department, and the Radiation Center at Oregon State University.

Initial attempts have shown that appreciable changes take place in sediments as noted by the increase in both the soluble orthophosphate and the soluble organic phosphorus concentration of interstitial water (Table V).

FWPCA EXTRAMURAL RESEARCH PROGRAM RELATING TO SEDIMENT-WATER NUTRIENT INTERCHANGE

This paper has described some of the work carried out by the National Eutrophication Research Program at Corvallis, Oregon, by three men during the past 22 months. Coordinated with this program are several research grants, demonstration grants, and research contracts (Table VI) which have been in progress part of the year between July 1, 1968 and July 1, 1969.

CONCLUSIONS

Quantitative measurement of the overall nutrient interchange process between sediments and overlying lake water is difficult at present using either field or laboratory tests.

An attempt to determine whether nutrients are available in lake sediments has been determined by analysis of interstitial water from sediments taken from the highly eutrophic Agency and Upper Klamath lakes in Oregon and others in the western part of the United States. In these lakes much soluble phosphorus and nitrogen (as ammonia) is available for algal growth when the sediment is somehow physically mixed with the overlying water. In shallow lakes, such as Upper Klamath, Salton Sea, and Lake Herman, there are ample opportunities for this to occur by resuspension of sediments from wave action caused by wind over the lake, stirring by benthic organisms, fish, boating, gas release from the sediment, lifting of the sediment by algal growths

TABLE V

CHANGE OF PHOSPHORUS COMPOSITION IN INTERSTITIAL WATER AS A RESULT OF STERILIZATION OF UPPER KLAMATH LAKE SEDIMENTS BY COBALT IRRADIATION

	P Ortho mg/ℓ	P Soluble Organic mg/ℓ	P Total Soluble mg/ℓ
Buck Island			
Analysis of Interstitial Water Before Irradiation	0.2	-	-
Analysis after Irradiation (6 mega rads)	4.1	11.4	15.5
Howard Bay			
Analysis of Interstitial Water Before Irradiation	9.5	0	9.5
Analysis after Irradiation (5 mega rads)	33	4	37
Howard Bay			
Analysis of Interstitial Water Before Irradiation	7.0	0.2	7.2
Analysis after Irradiation (3 mega rads)	31.5	16	48
Buck Island			
Analysis of Interstitial Water Before Irradiation	0.31	0.06	0.37
Analysis after Irradiation[a] (1 mega rad)	7.0	1.0	8.0

[a] Sample not sterile after irradiation.

to the lake surface, and ordinary diffusion processes. In cores it has been found that there are considerable concentrations of nutrients in the interstitial water at levels 2 to 6 ft below the sediment-water interface. This information may be useful in predicting the value of dredging as a method for lake restoration.

Sediments from the lakes studied change appreciably upon freezing and storage. Only fresh sediments give accurate results if soluble nutrients in interstitial water are to be determined.

Irradiation of the sediments with cobalt (1 to 6 mega rads) for sterilization was found to increase the soluble orthophosphate and soluble organic compounds in the interstitial water.

TABLE VI

FWPCA EXTRAMURAL RESEARCH PROGRAM RELATING TO SEDIMENT-WATER NUTRIENT INTERCHANGE

	Title	Grantee	Principal Investigator
Research Contract	An Investigation of the Release of Phosphorus from Lake Sediments	Battelle-Northwest Richland, Wash.	R. E. Wildung
Demonstration Grants	A Study to Evaluate the Effect of Silt and Silt Removal in a Northern Prairie Lake	General Beadle State College, Madison, S. Dak.	C. K. Brashier
	Effects of Domestic Pollution Abatement on a Eutrophic Lake	Univ. of Notre Dame	M. W. Tenney
Research Grants	The Role of Phosphorus–Sediment Interactions in Eutrophication	Univ. of Wisconsin	D. E. Armstrong
	Nitrogen Transformations in Lake Sediments	Univ. of Wisconsin	D. R. Keeney
	Sediment-Water-Bacteria Interaction in Eutrophication	Oregon State Univ.	R. E. Pacha
	Phosphate Exchange with Sediments	Harvard Univ.	W. Stumm
	Metal Ion Complexing in Lake Waters	Rensselaer Polytechnic Inst.	R. A. Bailey

Laboratory experiments indicate that the Upper Klamath Lake sediments promote algal growth.

Laboratory experiments for elimination of sediment nutrient release by addition of chemicals which form hydrous oxides and insoluble phosphates indicate that this technique for lake restoration may provide only temporary relief.

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THE SHAGAWA LAKE, MINNESOTA, EUTROPHICATION RESEARCH PROJECT

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The Shagawa Lake Eutrophication Research Project, located at Ely, Minnesota, is part of the National Eutrophication Research Program based at the Pacific Northwest Water Laboratory, Corvallis, Oregon, Federal Water Pollution Control Administration, Department of the Interior. The ultimate aim of the project is to determine whether Shagawa Lake would be likely to recover from its presently advanced state of eutrophication if secondary sewage effluent presently entering the lake were changed to nutrient-stripped tertiary. In order to demonstrate this, a pilot tertiary treatment plant and a series of in-lake floating experimental basins have been put into operation by the project.

Ely, with a population of 5,500, is located in northeastern Minnesota about 110 miles northwest of Duluth and a few miles south of the Canadian border. Shagawa Lake is partially surrounded by the city and was chosen as the site for experimental work for the following reasons: 1) It had a history of algal blooms; 2) The lake had received domestic sewage effluent for many years, 3) There was very little agriculture in the area and no industry (except for iron mining); hence, practically all pollution entering the lake was from municipal sewage; and 4) The eutrophic state of the lake was uncommon among the many lakes in northeastern Minnesota. The quality of the lake is particularly important because its outflow passes through parts of the Superior National Forest, the Boundary Waters Canoe Area, and Canada. The U. S. Forest Service is particularly interested in maintaining high water quality in the Boundary Waters Canoe Area.

Shagawa Lake is in the Hudson Bay – Rainy Lake drainage area. Much of the lake basin is covered with coniferous-deciduous forest. The lake has an area of 2,337 acres (946 ha), a mean depth of 22 feet (6.7 m), a maximum depth of 48 feet (14.6 m), and a normal capacity of 45,000 acre feet (5,553,000 m^3). It has a retention time of about one year.

Ely has had a municipal sewage system since about 1901. Treatment was initiated in 1912 with the installation of two Imhoff tanks. In 1954 most of the present sewage treatment facilities, including a primary settling tank, a high-rate trickling filter, and a secondary settling tank were constructed. The sewage effluent has always been discharged to the lake, presently at a rate of about 1,000,000 gallons per day. About 85 percent of the city is connected to the municipal sewage treatment plant, and about 15 percent uses individual septic tank systems; most of this ultimately enters the lake.

Nutrient budgets on the surface inflow to the lake indicate about 70 percent of the total phosphorus, and about 50 percent of the total nitrogen are from the discharge of the municipal treatment plant. About 80 percent of the surface inflow water comes from the Burntside River and about 2 percent from the treatment plant.

The relative distribution of groups of algae in Shagawa Lake, and its inflow and outflow streams during the Summer of 1968 is shown in Table I. Algae samples were obtained with a Van Dorn bottle. They were taken near the mouths of the tributary streams and near the head of the outflowing Shagawa River. In Shagawa Lake samples were obtained at 5-foot (1.5 m) depth intervals from surface to bottom at 13 stations arranged on four cross-lake transects. All samples from each transect were composited and counts and identifications performed only on the composites. Samples were collected at 2-week intervals. Counting was by the "clump" method utilizing a Sedgwick-Rafter counting cell. These data show that the dominant algae in the lake and the Shagawa River were blue-greens. Diatoms were dominant in surface inflow streams.

TABLE I

AVERAGE PERCENTAGE OF ALGAL GROUPS IN
SHAGAWA LAKE AND STREAMS,
MAY 26-SEPT. 27, 1968[a]

	Bluegreen	Green	Diatoms	Other
Shagawa Lake	48.3	22.6	25.5	3.5
Outlet				
Shagawa River	61.0	10.9	20.5	7.4
Inflow Streams				
Burntside River	10.7	32.5	41.9	14.8
Burgo Creek	20.3	29.7	43.2	6.8
Armstrong Creek	12.8	16.1	60.0	11.1
Longstorff Creek	3.4	23.8	69.4	3.4

[a] Based on Total Live Algae

Table II shows the average algal counts and algal dry weights in the lake, in the principal surface inflow streams, and in the outlet during the Summer of 1968. Both Shagawa Lake and the outlet were appreciably higher in algal counts and dry weight than any of the inflow streams.

TABLE II

ALGAL DRY WEIGHTS AND TOTAL COUNTS IN SHAGAWA LAKE AND STREAMS FROM JUNE THROUGH SEPTEMBER 1968

	Average Count No. /mℓ	Average Dry Weight mg/ℓ
Lake	1426	3.1
Inflow Stream		
Burntside River	100	2.3
Burgo Creek	44	1.8
Armstrong Creek	112	1.6
Longstorff Creek	89	2.1
Outlet		
Shagawa River	11655	6.9

Averages for total phosphorus and total nitrogen from Shagawa Lake, Shagawa River (outlet), Burntside River (inlet), and the sewage treatment plant effluent from July 1967 to January 1969 are shown in Table III. Samples were obtained in the same manner as described for algae.

TABLE III

AVERAGE PHOSPHORUS AND NITROGEN LEVELS, mg/ℓ, JULY 1967-JANUARY 1969

	Total Phosphorus-P	Total Nitrogen-N
Shagawa Lake	0.04	1.02
Shagawa River (outlet)	.11	2.00
Burntside River (inlet)	.02	.51
Municipal Sewage Treatment Plant Effluent	4.79	15.5

A pilot plant for tertiary treatment of effluent from the secondary municipal plant is an integral part of the Shagawa Lake project. It has a capacity of 20,000 (75,700 ℓ) gallons per day. The plant may be considered to have four unit processes. The first employs coagulation, settling and filtration; the second, activated carbon; the third, cation exchange; and the fourth, anion exchange. The first processing unit uses approximately 150 mg/ℓ of alum and 0.5 mg/ℓ of a polyelectrolyte floccing agent.

The feed stream to the pilot plant is from the effluent of the municipal sewage treatment plant taken just before chlorination.

Performance through the several unit processes of the pilot plant is shown in Table IV. These data are averages representing about six weeks operation and about 500,000 gallons (1,892,500 ℓ) of flow-through. The data show 99.1 percent removal of total phosphorus in the chemical coagulation, settling, filtration (CF) process and 99.8 percent cumulative removal of phosphorus through all units of processing. Except for an approximate 75 percent removal of iron, the CF unit did not remove appreciable quantities of dissolved materials. The cumulative removal of all components through all processing units of the pilot plant was high. Very little nitrogen was removed through the CF unit, but complete pilot plant processing removed about 85 percent of the total nitrogen. Turbidity of effluent from the CF unit was consistently less than 0.5 standard Jackson units.

G. P. Fitzgerald, University of Wisconsin, has evaluated pilot plant treated sewage effluent in algal growth potential tests* using Chlorella pyrenoidosa (Wis. 2005) and Microcystis aeruginosa (Wis. 1036). Effluent from the coagulation, flocculation, filtration unit (CF) appeared to be phosphorus-limiting for algal growth in 38 out of 40 samples tested. Phosphorus was limiting in all samples from carbon treatment (CC), decationization (CE), and deanionization (AE) units. In general, less algal growth was attained in the CC and CE samples than in the CF samples, and significantly less in AE samples. Note that treatment units were arranged in the order CF, CC, CE, AE, and samples from any unit had also gone through each preceding unit. Fitzgerald's results showed that nitrogen was the second limiting factor for algal growth after P in CC, CE, and AE treated samples.

The stimulatory effect of effluent from the tertiary pilot plant, and from the municipal secondary plant, upon the growth of algae in Shagawa Lake is being studied through the use of in situ floating test basins on three size scales. Originally, three 150,000 gallon (567,750 ℓ) capacity basins, fabricated of nylon coated with neoprene, were acquired for this purpose. The large size of the basins (40 x 40 ft [12.2 x 12.2 m] at the surface, 5 x 5 ft [1.5 m] at the bottom, 20 ft [6.1 m] depth) was believed to aid in inhibition of side-wall effects in algae growth experiments. Placement in the lake provides temperature, surface light, air environment, and motion similarity with the lake.

Experimental work to determine the relative algal growth stimulating effects of secondary sewage effluent (SE) and tertiary sewage effluent (CF, AE) when added to lake water are being carried out in the 150,000 gallon (567,750 ℓ) basins using lake water control mixtures.

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*Fitzgerald, G. P., "Final Report on Algal Growth Potential of Treated Ely, Minnesota, Sewage Samples," October 28, 1968.

TABLE IV

ANALYTICAL VALUES[a], mg/ℓ, OF EFFLUENT FROM TERTIARY SEWAGE TREATMENT PILOT PLANT

	Sample Source[b]						
Analysis	SE	CF	Cumulative % Removal	CE	CC	AE	Cumulative % Removal
Total PO_4-P	5.6	0.05	99.1	0.05	0.04	0.01	99.8
Ortho PO_4-P	4.9	0.04	99.2	0.02	0.02	< 0.01	99.8+
Kjeldahl-N	16.3	16.3	0	3.7	2.8	2.6	84
NH_3-N	13.0	11.4	12.3	0.6	0.3	0.3	97.7
NO_3-N	1.2	1.3	none	0.6	0.06	0.05	95.8
Na	27.3	30.9	none	2.2	2.5	1.5	94.5
K	8.0	7.0	12.5	0.5	0.5	0.4	95
Fe	0.8	0.2	75	-	-	0.03	96.2
Sol. SiO_2	8.1	7.3	9.9	7.7	7.7	0.4	95
TDS	217	269	-	186	177	19	91.2
Total Hard.	94.5	101.4	-	0	0	0	100
Ca Hard.	76.6	83.7	-	0	0	0	100

[a] Averages from 6 weeks operation — approximately 500,000 gallons through flow.

[b] SE = effluent from trickling filter sewage plant.

CF = effluent from chemical-coagulation-filtration step effluent.

CC = effluent from carbon column effluent.

CE = effluent from cation exchange effluent.

AE = effluent from anion exchanger
(this has been processed in all steps of the pilot plant).

One hundred gallon (378 ℓ) and eight gallon (30 ℓ) floating basins are being used to determine the algal productivity of mixtures of tertiary and secondary sewage effluent with Burntside River water. The Burntside is the major tributary to Shagawa Lake, providing about 80 percent of the entering surface flow. The composition of the lake at some future time, therefore, were tertiary treatment applied to the entire output of municipal sewage, would be essentially a mixture of Burntside River water and tertiary effluent. It is necessary to conduct experiments utilizing mixtures of Burntside water and variously treated sewage effluents in order to obtain some predictive capability as to possibilities for restoration of the lake through tertiary sewage treatment.

Preliminary data only are available at this time. Figure 1 shows chlorophyll a analyses plotted against time for an experiment carried out under static conditions in the three large basins containing 95 percent Shagawa Lake water and 5 percent filtered Shagawa Lake water (Basin E), 5 percent tertiary water treated through the CF stage only (Basin M), and 5 percent secondary sewage effluent (Basin W). These data indicate that the standing crops of algae, as measured by chlorophyll a, were very appreciably greater in the mixture containing 5 percent SP than those developed in mixtures containing 5 percent CF or 5 percent filtered lake water.

Changes in orthophosphate concentration with data for the same experiment are shown in Figure 2. These data indicate slight changes in the CF and filtered lake water mixtures corresponding to relatively small changes in chlorophyll a, and a steady decline in the SP mixture corresponding to relatively large increases in chlorophyll a (Figure 1). A leveling off in change in orthophosphate concentration occurred with the decline in chlorophyll a concentration. The declines in chlorophyll a concentration occurred approximately at the same time in all three experimental basins.

Figure 3 shows changes in chlorophyll a with time in the three 100 gallon (378 ℓ) basins. An appreciable stimulation to algal growth from the addition of 2 percent SP to 97 percent Burntside River water is apparent. Considerably greater growth was evident in this mixture compared to Shagawa Lake water, or mixtures of 97 percent Burntside River water with 2 percent CF or AE. The indicated chlorophyll increase in the AE basin appears to be anomalous; it did not occur in recent repeats of this experiment.

Orthophosphate concentrations corresponding to the chlorophyll a measurements in the 100 gallon (378 ℓ) basins are shown in Figure 4. The mixture containing SE was considerably higher and varied inversely with chlorophyll a concentration.

Figure 5 shows chlorophyll a data for an experiment involving the 8 gallon (30 ℓ) basins which was identical to and carried out concurrently with the experiment described for the 100 gallon (378 ℓ) basins. Results were generally similar.

The data presented here are incomplete and conclusions are preliminary. However, the following appear to be indicated:

1. Lake water containing 5 percent secondary sewage effluent had appreciably higher algal productivity than Shagawa Lake water only or Shagawa Lake water containing 5 percent tertiary sewage effluent (CF).

2. Shagawa Lake water containing 5 percent tertiary sewage effluent (CF) was not appreciably different from lake water only.

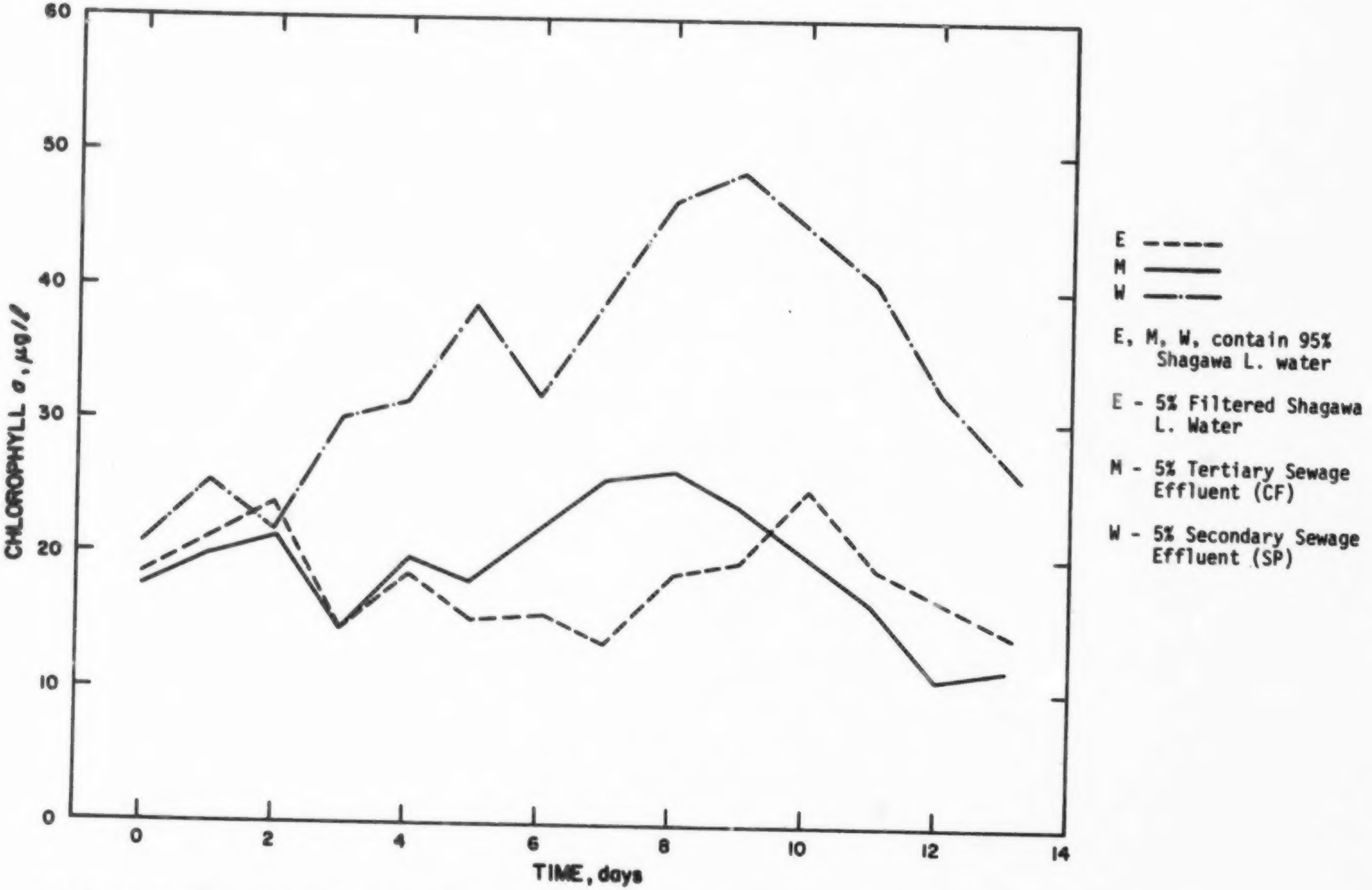


FIGURE I. LARGE BASIN (150,000 gallon) BATCH-TYPE EXPERIMENT WITH CHLOROPHYLL *a*

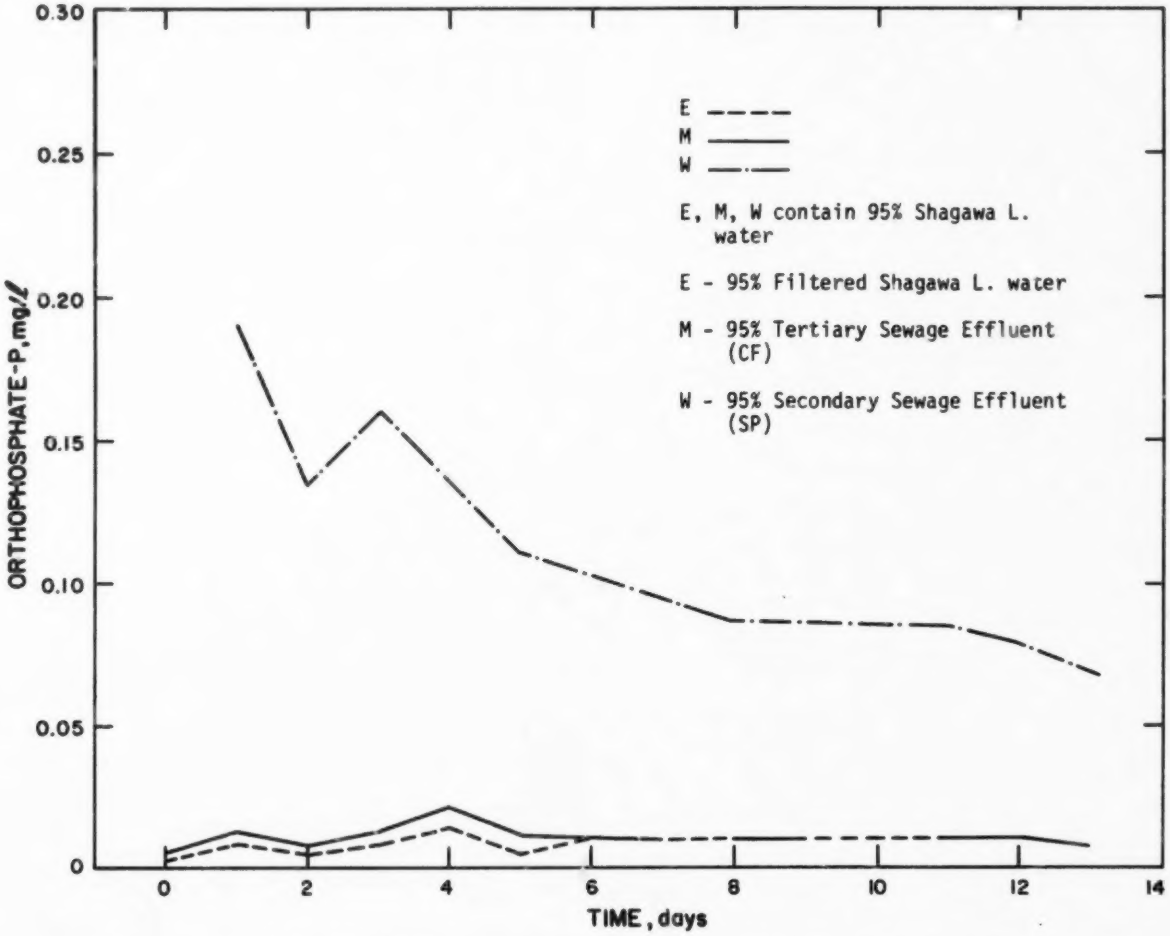
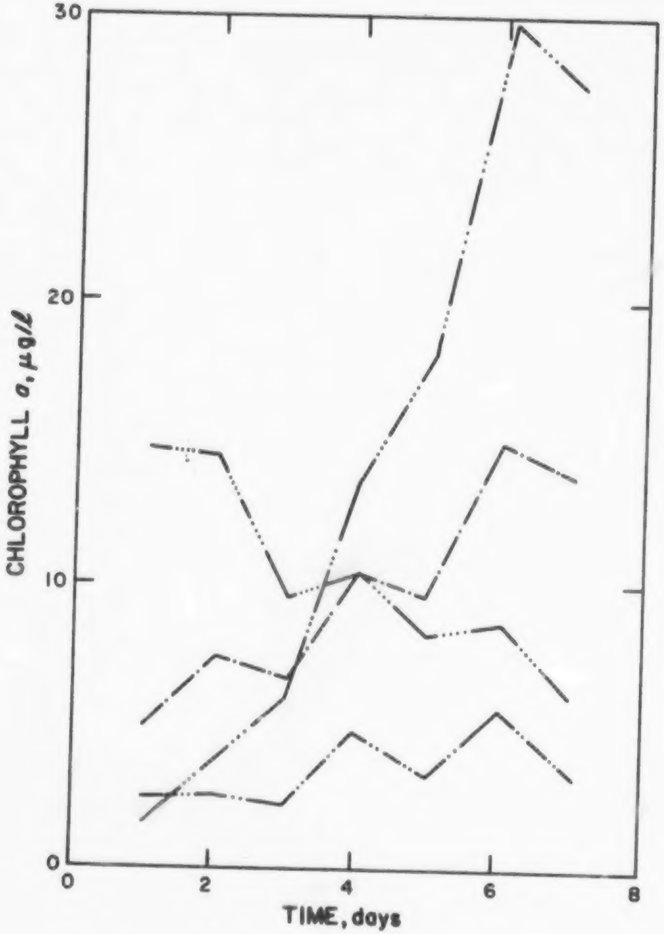


FIGURE 2. LARGE BASIN (150,000 gallon) BATCH-TYPE EXPERIMENT WITH ORTHOPHOSPHATE-P



Basin 1
Basin 2
Basin 3
Basin 4

Basin 1

97% Burnt. R., 2% AŁ, 1% Unfilt. Shagawa L.

Basin 2

97% Burnt. R., 2% CF, 1% Unfilt. Shagawa L.

Basin 3

97% Burnt. R., 2% Sec. (SP), 1% Unfilt. Shagawa L.

Basin 4

99% Filt. Shagawa L., 1% Unfilt. Shagawa L.

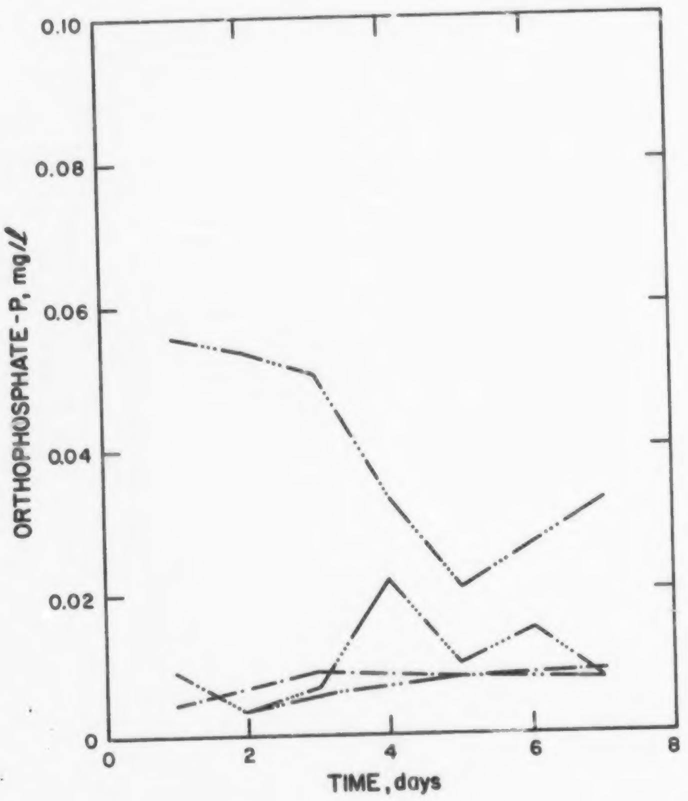
Basin 5

99% Burnt. R., 1% Unfilt. Shagawa L.

NOTE: Basin 5 lost on second day

Day	Chlorophyll a
1	2.2
2	2.6

FIGURE 3. INTERMEDIATE BASIN (100 gallon) BATCH-TYPE EXPERIMENT WITH CHLOROPHYLL *a*



Basin 1

97% Burnt. R., 2% AE, 1% Unfilt. Shagawa L.

Basin 2

97% Burnt. R., 2% CF, 1% Unfilt. Shagawa L.

Basin 3

97% Burnt. R., 2% Sec. (SP), 1% Unfilt. Shagawa L

Basin 4

99% Filt. Shagawa L., 1% Unfilt. Shagawa L.

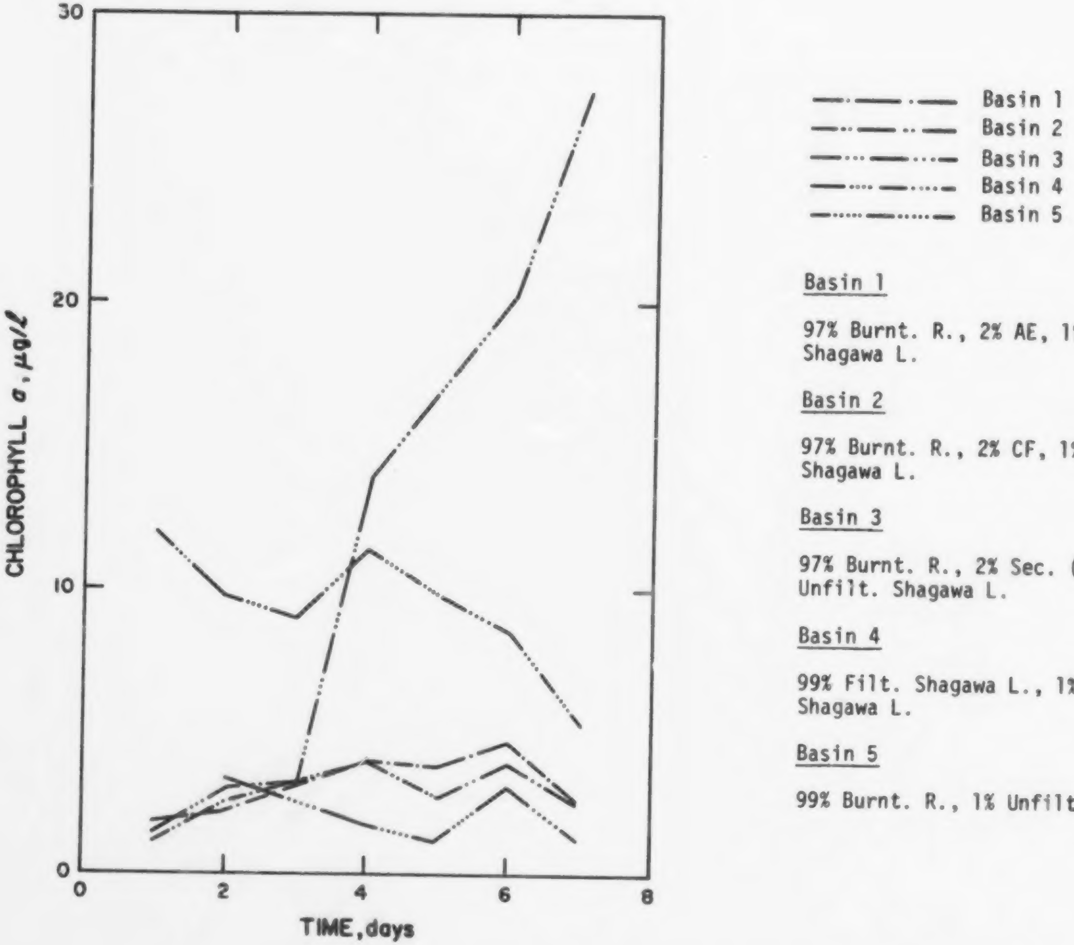
Basin 5

99% Burnt. R., 1% Unfilt. Shagawa L.

NOTE: Basin 5 lost on second day

Day	Chlorophyll a
1	2.2
2	2.6

FIGURE 4. INTERMEDIATE BASIN (100 gallon) BATCH-TYPE EXPERIMENT WITH ORTHOPHOSPHATE-P



Basin 1

97% Burnt. R., 2% AE, 1% Unfilt. Shagawa L.

Basin 2

97% Burnt. R., 2% CF, 1% Unfilt. Shagawa L.

Basin 3

97% Burnt. R., 2% Sec. (SP), 1% Unfilt. Shagawa L.

Basin 4

99% Filt. Shagawa L., 1% Unfilt. Shagawa L.

Basin 5

99% Burnt. R., 1% Unfilt. Shagawa L.

FIGURE 5. SMALL BASIN (8 gallon) BATCH-TYPE EXPERIMENT WITH CHLOROPHYLL *a*

3. Burntside River water containing 2 percent secondary sewage effluent had very appreciably higher algal productivity than corresponding mixtures containing tertiary effluent.

Further testing of Shagawa Lake water and Burntside River water is continuing throughout the summer using other concentrations of additives, in order to more completely explore the biostimulation achieved through sewage fertilization of these primary water sources. In addition, experiments will be carried out in which various concentrations of nitrogen and phosphorus are added to Burntside River water, to determine whether either or both of these two elements act to limit algal production. It is anticipated that results of these series of experiments will provide information necessary to decisions on future management policies for Shagawa Lake.

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